

TARGET DETECTION USING TEMPERATURE CONTROLLED PROBES

Technical Field

The present invention relates generally to the field of molecular biology. More specifically, the present invention provides oligonucleotides and methods for their use in the detection and/or differentiation of targets. The oligonucleotides and methods find particular application in amplifying, detecting, discriminating and/or quantifying multiple targets simultaneously.

Incorporation by Reference

The present application claims priority from Australian provisional application number 2023901468 filed on 15 May 2023, the entire contents of which are incorporated herein by reference.

Background

Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

Genetic analysis is routinely used in the clinic for assessing disease risk, diagnosis of disease, predicting a patient's prognosis or response to therapy, and for monitoring a patient's progress. The introduction of such genetic tests depends on the development of simple, inexpensive, and rapid assays for discriminating genetic variations.

Methods of *in vitro* nucleic acid amplification have wide-spread applications in genetics and disease diagnosis. Such methods include polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), quantitative polymerase chain reaction (qPCR), strand displacement amplification (SDA), nicking enzyme amplification reaction (NEAR), helicase dependent amplification (HDA), Recombinase Polymerase Amplification (RPA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), transcription-mediated amplification (TMA), self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), Ligase Chain Reaction (LCR) or Ramification Amplification Method (RAM). Most of these target amplification strategies requires the use of oligonucleotide primer(s). In most protocols, the process of amplification results in the accumulation of amplicons which

incorporate the oligonucleotide primers at their 5' termini of each strand, and which contain newly synthesized copies of the sequences located between the primers.

One method for monitoring the accumulation of amplicons in real-time, or at the conclusion of amplification, involves detection using PlexZymes, also known in the literature as Multi-component Nucleic Acid Enzymes or MNAzymes. PlexZymes have been previously described to cleave probes which are dual-labelled universal substrates which have either a linear or a hairpin conformation. Linear substrates were the first type of reporter probes described for MNAzymes/PlexZymes and may be considered as "standard" substrates or probes. Hairpinned PlexZyme substrates are known in the art as either LOCS (Loops Conected to Stems) Probes or PlexPlus Probes. LOCS probes have specific features which allow control of signal generation via manipulation of temperature. Other methods, systems and probe types for monitoring the accumulation of amplicons in real-time, or at the conclusion of amplification, include target-specific Molecular Beacons, Sloppy Beacons, Binary DNA probes (also known as universal Molecular Beacons), Eclipse probes, TaqMan Probes or Hydrolysis probes, Scorpion Uni-Probes or Bi-Probes, Catchers and Pitchers for TOCE technology, Dual Hybridization probes, Double-stranded probes (Yin-Yang probes) and/or the use of intercalating dyes such as SYBR Green.

Melt curve analysis can be performed during or at the conclusion of several of these protocols to obtain additional information since double stranded nucleic acid molecules with different sequences denature at different temperatures, known as the melting temperature or T_m . Such protocols measure melting curves which result from either a) the separation of the two strands of double stranded amplicons in the presence of an intercalating dye, or b) the separation of one strand of the amplicon and a complementary target-specific probe labelled with a fluorophore and quencher or c) separation of non-target related duplexes, for example, Catcher duplexes which are only generated in the presence of target. Melt curve analysis provides information about the dissociation kinetics of two nucleic acid strands during heating. The melting temperature (T_m) is the temperature at which 50% of the double stranded nucleic acid molecules have dissociated. The T_m is dependent on the length, sequence composition and G-C content of the paired nucleotides. Elucidation of information about the target DNA or RNA from melt curve analysis conventionally involves a series of fluorescence measurements acquired at small temperature intervals, typically over a broad temperature range. In some protocols the derivative of this curve is then plotted as a function of temperature to obtain the melt curve. Melting temperature does not only depend upon on the sequence of the nucleic

acid strands. The T_m can be influenced by many factors including the type of nucleic acid (DNA, RNA, LNA or others), the concentrations of oligonucleotides, cations in the buffer (both monovalent (Na^+) and divalent salts (e.g. Mg^{2+})), and/or the presence or absence of destabilizing agents such as urea or formamide.

Melt curve analysis protocols are often slow and typically take between 30-60 mins to complete. Furthermore, melt curve analyses can require interpretation by skilled personnel and/or the use of specialised software for results interpretation. Hence, there is a high demand for faster and/or less complex alternatives to melt curve analyses. Further, melt curves are typically analysed post-PCR and therefore only allow for a qualitative determination of the presence or absence of a target in a sample. In many instances, a quantitative, or semi-quantitative, determination of the amount of genomic material present in a sample is required. Therefore, there is a high demand for fast alternatives to melt curve analysis that also provide quantitative information about a sample.

Hairpin probes or Stem-Loop probes have proven to be useful tools for detection of nucleic acids and/or monitoring target amplification. One type of hairpin probe, which is dual labelled with a fluorophore and quencher dye pair, is commonly known in the art as a Molecular Beacon. In general, these molecules have three features; 1) a Stem structure formed by hybridization of complementary 5' and 3' ends of the oligonucleotide; 2) a loop region which is complementary to the target, or target amplicon, to be detected; and 3) a fluorophore quencher dye pair attached at the termini of the Molecular Beacon. During PCR, the loop region binds to the amplicons due to complementarity and this causes the stem to open thus separating the fluorophore quencher dye pair. An essential feature of Molecular Beacons is that the loop regions of these molecules remain intact during amplification and are neither degraded or cleaved in the presence of target or target amplicons. The separation of the dye pair attached on the termini of an open Molecular Beacon causes a change in fluorescence which is indicative of the presence of target to which it is hybridized. The method is commonly used for multiplex analysis of multiple targets in a single PCR test. In general, for multiplex analysis, each Molecular Beacon has a different target-specific loop region and a unique fluorophore, such that hybridization of each different Molecular Beacons to each amplicon species can be monitored in a separate channel i.e. at a separate wavelength. A disadvantage of Molecular Beacons is that careful design and reaction temperature optimization is required to balance the transition between the hairpin conformation and the linear conformation adopted when the Molecular Beacons bind to the target.

The concept of Molecular Beacons has been extended in a strategy known as Sloppy Beacons. In this protocol the loop region of a single Beacon is long enough such that it can tolerate mismatched bases and hence bind to a number of closely related targets differing by one or more nucleotides. Following amplification, melt curve analysis is performed and different target species can be differentiated based on the temperature at which each of the duplexes formed by hybridization of the target species with the loop region of a Sloppy Beacon separate (melt). In this way multiple closely related species can be detected at a single wavelength and discriminated simultaneously by characterising the melting profile of specific targets with the single Sloppy Beacon. Standard Molecular Beacons and Sloppy Beacons differ from TaqMan and Hydrolysis probes in that they are not intended to be degraded or cleaved during amplification. A disadvantage of DNA hybridisation-based technologies such as sloppy beacons and TOCE is that they may produce false positive results due to non-specific hybridisation between probes and non-target nucleic acid sequences.

In general, the number of available fluorescent channels capable of monitoring discrete wavelengths limits the number of targets which can be detected, and specifically identified, in a single reaction on a fluorescent reader. Recently, three protocols have been described which allow detection and quantification of two or more targets at a single wavelength. The first protocol, known as “Tagging Oligonucleotide Cleavage and Extension” (TOCE), uses Pitcher and Catcher oligonucleotides. Pitchers have two regions, the Targeting Portion, which is complementary to the target, and the Tagging portion which is non-complementary and located at the 5' terminus. The Catcher oligonucleotide is dual labelled and has a region at its 3' end which is complementary to the tagging portion of the Pitcher. During amplification, the Pitcher binds to the amplicons and when the primers extend the 5'-3' exonuclease activity of the polymerase can cleave the Tagging portion from the Pitcher. The released Tagging portion then binds to the Catcher Oligonucleotide and functions as a primer to synthesise a complementary strand. The melting temperature of the double stranded Catcher molecule (Catcher-T_m) then acts as a surrogate marker for the original template. Since it is possible to incorporate multiple Catchers with different sequences and lengths, all of which melt at different temperatures, it is possible to obtain a series of Catcher-T_m values, indicative of a series of targets whilst still measuring at a single wavelength. Limitations with this approach include inherent complexity as it requires the released fragment to initiate and complete a second extension on an artificial target, and post amplification analysis of multiple targets requires complex algorithms to differentiate or quantify the proportion of signal related to

each specific target. The method measures fluorescence at various temperatures, however at the lowest temperature all double stranded Catcher molecules fluoresce giving a combined signal for all targets. At the highest temperature where fluorescence is acquired, only one double stranded Catcher molecule, which has the highest T_m , will remain double stranded and hence will fluoresce. An algorithm is then required to determine the contribution from the one or more targets detected in the reaction.

In the second protocol, multiple LOCS reporters, can be combined in the same reaction to measure multiple targets at a single wavelength. Intact LOCS reporters or probes contain a stem region, labelled with a fluorophore quencher dye pair at each terminus, and a Loop region which comprises a substrate for an enzyme. In the presence of a target, the substrate can be cleaved or hydrolyzed resulting in Split LOCS structures. Enzymes suitable for mediating target-dependent substrate modification include catalytic nucleic acids such as an PlexZymes and DNAzymes, or protein enzymes such as exonucleases or endonucleases. The melting temperatures of the stem regions of Intact LOCS (Intact LOCS T_m) are higher than those of resultant Split LOCS (Split LOCS T_m) since intramolecular bonds are stronger than intermolecular bonds. Both the Intact LOCS and the Split LOCS will be either quenched, or will generate fluorescence, depending upon whether the temperature of the reaction milieu is above or below the melting temperature of their stems. The presence of fluorescence at temperatures below the Intact LOCS T_m but above the Split LOCS T_m is indicative of the presence of the target which facilitates the cleavage. The target can be directly detected, or amplicons produced by target amplification protocols, can be detected. Multiple LOCS reporters can be combined to facilitate detection of multiple targets at a single wavelength in a single reaction. In such reactions, all LOCS are labelled with dyes that can be monitored at the same wavelength; however, each has a stem designed to melt at a different temperature, and each has a loop that is cleavable only in the presence of its specific target. In real time PCR, fluorescence specific for two LOCS/two targets can be acquired at two temperatures where a lower temperature is set to be suitable for measuring fluorescence associated with the Split LOCS with the lower T_m stem only. A second higher temperature is suitable for measuring the combined signal for both high and low T_m Split LOCS but will not generate fluorescence from either LOCS probe when Intact.

Recently a third approach has been described which allows detection of multiple targets at a single wavelength in real time without the need for an algorithm to determine the contribution from the one or more targets. The method combines LOCS reporters with other probe types, for example Molecular Beacons in single reactions where probe pairs

are labelled with the fluorophores that produce fluorescence in the same channel. In one format at the first detection temperature, the Molecular Beacon can bind to a first target, causing spatial separation of the fluorophore and quencher and an increase in fluorescence. At a second higher detection temperature, at which it can neither bind to its target, nor form a quenched hairpin, the Molecular Beacon can adopt a random coil structure, which only contributes to the constant level of background fluorescence that is present regardless of the presence or absence of the first target. The LOCS reporter is designed to be cleaved in the presence of a second target and to have a Split LOCS T_m that is above the first detection temperature but below the second detection temperature. As such, at the first temperature the LOCS reporter remains quenched regardless of the presence or absence of the second target but causes increased fluorescence at the higher temperature if there has been target dependent cleavage of Intact LOCS. Overall, the Molecular Beacon is fluorescent at the first lower detection temperature if the first target is present, and the LOCS is quenched regardless of the presence or absence of the second target; whilst at the second, higher detection temperature the Molecular Beacon contributes to background fluorescence at a constant level, which is unaffected by the presence or absence of the first target and the LOCS is fluorescent only if the second target is present. As such each temperature measures changes in fluorescence associated with only one type of probe in the presence of a target and hence no algorithm is required to ascertain a signal specific for each target and probe type. Whilst this protocol is advantageous and flexible, it uses Molecular Beacons or other probe types which often have restrictive design requirements with respect to temperature; and/or may involve the use of specific reagents including, for example, polymerases which lack exonuclease activity.

A need exists for improved compositions and methods for the simultaneous detection, differentiation, and/or quantification of multiple unique amplicons generated by PCR or by alternative target amplification protocols at a single wavelength.

It is an object of the present invention to overcome or ameliorate at least one of the disadvantages of the prior art, or to provide a useful alternative.

Summary of the Invention

Provided herein are methods and compositions which extend the capacity for multiplex analysis of targets. The present invention relates to methods and compositions for the detection and quantification of multiple targets at a single wavelength. These

methods and compositions employ Multiple-component Temperature-controlled Probes herein referred to as M-Tec Probes. By way of example, M-Tec Probes are multiple-component complexes composed of at least two oligonucleotide components wherein a first oligonucleotide component (OC1) connected to a first detection moiety is capable of being modified by an enzyme only in the presence of a specific target and a second oligonucleotide component (OC2) is labelled with a second detection moiety. The first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a double-stranded portion. The region or position which is amenable to enzymatic modification is located on OC1 between the first capture region and the first detection moiety. In some examples, OC1 is not directly labelled with more than one detection moiety. The first and second oligonucleotide components are capable of hybridising together at temperatures below the melting temperature (T_m) of the double-stranded portion (T_m OC1/OC2). The first oligonucleotide component is either directly labelled with the first detection moiety, or alternatively has a second region of complementarity with a third oligonucleotide component (OC3) which is directly labelled with a first detection moiety. If present, a third oligonucleotide component is capable of hybridization with the first oligonucleotide component at temperatures below the T_m of the complementary regions (T_m OC1/OC3). If this temperature is greater than the T_m OC1/OC2, then at temperatures below T_m OC1/OC2 all oligonucleotide components in a complex will be hybridized. The first and second detection moiety may be, for example, a fluorophore and a quencher or vice versa.

When all oligonucleotide components are hybridized, and the first oligonucleotide component is unmodified, the fluorophore and quencher are in close proximity resulting in intact M-Tec Probe complexes which are quenched. In the presence of the target that the M-Tec Probe is designed to detect, a sensor region of the first oligonucleotide component is modified, for example by cleavage or hydrolysis by an enzyme. Enzymatic modification of the sensor region of the first oligonucleotide component generates a first fragment comprising the first capture region and a second fragment connected to the first detection moiety, thereby enabling the first and second detection moieties to spatially separate and generate a first detectable signal. The first detectable signal can be distinguished from background signal under the conditions of measurement. In other words, modification of the sensor region causes separation of the first capture region from the region of the first oligonucleotide component connected to the first detection moiety. Where the present disclosure refers to two detection moieties spatially separating and generating a detectable signal, it will be understood that the detection moieties spatially

separate from each other which thereby enables one of the detection moieties to generate a detectable signal. Where the present disclosure refers to first and second detection moieties being capable of generating a detectable signal, it will be understood that only one of the detection moieties (i.e., either the first or the second detection moiety) need emit a detectable signal, and that the emission is detectable due to the spatial separation of both detection moieties. In that sense, the first and second detection moieties are capable of generating a detectable signal, even though only one of the moieties emits the detectable signal. For example, the first detection moiety may be a fluorophore and second detection moiety may be a quencher; both detection moieties are capable of generating a detectable signal by spatial separation such that the fluorophore emits a detectable fluorescence signal. In some examples, the first and second detection moieties permanently or irreversibly separate, generating a detectable signal that is detectable within a defined temperature range. By way of example, the modification of the sensor region occurs at a position between the detection moieties such that it causes separation of the fluorophore and the quencher. Resultant target-dependent detectable signals, for example increases in fluorescence, can be measured at temperatures below T_m OC1/OC2. At temperatures above the T_m OC1/OC2, the first oligonucleotide component, or first fragment thereof, dissociates from the second oligonucleotide component, and the detection moieties are separated. The resulting fluorescence contributes to the background fluorescence observed, in a manner which is constant regardless of the presence or absence of the target that the M-Tec probe is designed to detect. At this temperature, the second oligonucleotide component will no longer hybridize to either the unmodified first oligonucleotide component in reactions where no target is present, or to a hydrolysed/cleaved first fragment of the first oligonucleotide component, which has been modified in the presence of target.

In this manner M-Tec Probes will generate target dependent increases in fluorescence at temperatures below T_m OC1/OC2 but no change in fluorescence will be observed, regardless of the presence or absence of target, at temperatures above the T_m OC1/OC2.

Various types of M-Tec Probes are disclosed and exemplified. One type of M-Tec Probe is suitable for modification/cleavage by a PlexZyme (i.e., an MNase). These probes, denoted herein as M-Tec-P probes, have a sensor region in the OC1 which can serve as a substrate for a PlexZyme. In some embodiments, an OC2 hybridizes to a capture region of the OC1 which does not hybridize/bind to the substrate binding arms of the PlexZyme. The M-Tec-P probe can bind to the substrate binding arms of a PlexZyme

when one assembles in the presence of its specific target. Cleavage of the sensor region of the OC1 of the M-Tec-P Probe results in generation of a first fragment and a second fragment of OC1 and thus in separation of the fluorophore and quencher. At temperatures below the T_m OC1/OC2, an increase in fluorescence is indicative of the presence of the target, which facilitated assembly of the PlexZyme. At temperatures above the T_m OC1/OC2, no change in fluorescence is observed regardless of the presence or absence of the target. As well as PlexZymes, M-Tec-P probes may also be cleaved by other types of DNA or RNA based enzymes, for example deoxyribozymes (i.e., DNAzymes), aptazymes or ribozymes. Since the mechanism of enzymatic cleavage is not mediated by a protein enzyme, these M-Tec-P probes can be used for direct detection of targets in reactions which do not encompass *in vitro* target amplification. In some embodiments, M-Tec-P probes can be used in detection protocols which are devoid of any protein enzymes.

Another type of M-Tec Probe is suitable for cleavage by exonuclease activity, for example, 5'-3' exonuclease of Taq polymerase, in the presence of target. In these probes, denoted herein as M-Tec-H probes, the sensor region of the OC1 includes a sequence which is complementary to the target to be detected. In some embodiments the OC2 hybridizes to a region of the OC1 which does not hybridize/bind to the target. During PCR, the OC1 binds to the target amplicons and is hydrolysed by the exonuclease activity of polymerase, resulting in generation of a first fragment and a second fragment and separation of the fluorophore and quencher. At temperatures below the T_m OC1/OC2, an increase in fluorescence is indicative of the presence of the target. At temperatures above the T_m OC1/OC2, no change in fluorescence will be observed regardless of the presence or absence of the target. In some embodiments hydrolysis of the first oligonucleotide component produces a first fragment which retains the capacity to form the first double stranded portion by hybridization to the second oligonucleotide component.

Another type of M-Tec Probe is suitable for cleavage by endonuclease activity, for example, a nicking endonuclease in the presence of target. In these probes, denoted herein as M-Tec-E probes, the sensor region of the OC1 includes a sequence which is complementary to the target to be detected. In some embodiments the OC2 hybridizes to a region of the OC1 which does not hybridize/bind to the target. The OC1 of the M-Tec-E probe can bind to the target and form a double stranded recognition site for a nicking enzyme. This enzyme can then cleave the OC1, resulting in generation of a first fragment and a second fragment and separation of the fluorophore and quencher, while leaving the target intact. At temperatures below the T_m OC1/OC2, an increase in fluorescence is indicative of the presence of the target. At temperatures above the T_m OC1/OC2, no

change in fluorescence will be observed regardless of the presence or absence of the target. In other scenarios, M-Tec probe can be cleaved by a restriction enzyme which cleaves both strands of a duplex. In some embodiments, M-Tec-E probes are suitable for use in methods where there is direct detection of the target i.e. in reactions that are not subjected to *in vitro* amplification. In other embodiments, M-Tec-E probes are suitable for use in conjunction with *in vitro* amplification. Isothermal *in vitro* amplification protocols may utilise any endonuclease compatible with the reaction temperature. When *in vitro* amplification involves thermocycling, for example PCR, thermostable endonucleases may be preferred. In some embodiments, a first fragment generated from cleavage of the OC1 by the endonuclease retains the capacity to form the first double stranded portion by hybridization to the second oligonucleotide component.

In some embodiments, M-Tec Probes are used in combination with LOCS Probes. In other embodiments, M-Tec Probes can be combined with other probe and substrate types well known in the art which include, but are not limited to, dual labelled linear PlexZyme substrates, TaqMan probes or Hydrolysis probes, Molecular Beacons, Sloppy Beacons, Eclipse probes, Scorpion Uni-Probes or Bi-Probes, Catcher/Pitcher Oligonucleotides, Double-stranded probes (Yin-Yang probes) and dual-hybridization probes.

The combination of a M-Tec Probe with other probe or substrate types allows greater multiplexing capacity, wherein multiple targets may be detected, identified and/or quantified at a single wavelength. By way of example, an M-Tec Probe, together with one or more LOCS probes, both of which incorporate the same detection moiety (e.g. the same fluorophore) may be used to individually discriminate multiple targets within a single reaction. The approach involves measurement of the signal generated from the probes at discrete temperatures. In some embodiments a first target is measured at a first temperature by monitoring any changes in fluorescence associated with modification of an M-Tec Probe and a second target is measured at a second temperature by monitoring any changes in fluorescence associated with modification of a LOCS probes.

The present disclosure relates to at least the following embodiments:

Embodiment 1. A method for determining the presence or absence of a target in a sample, the method comprising:

(a) preparing a mixture for a reaction by contacting the sample or a derivative thereof putatively comprising the target with:

- a multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,
 - wherein the first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a first double-stranded portion,
 - wherein the first oligonucleotide component further comprises a sensor region capable of serving as a substrate for an enzyme,
 - wherein the first oligonucleotide component is connected to a first detection moiety and the second oligonucleotide component is connected to a second detection moiety;
- an enzyme capable of modifying the sensor region of the first oligonucleotide component only when the target is present in the sample;
- (b) treating the mixture under conditions suitable for the enzyme to modify the sensor region of the first oligonucleotide component to thereby generate a first fragment comprising the first capture region and a second fragment connected to the first detection moiety, thereby enabling the first and second detection moieties to spatially separate and generate a first detectable signal,
- (c) measuring a level of background signal or detectable signal generated at a defined temperature at or below which the first capture region is hybridised to the second oligonucleotide component; and
- (d) determining the presence or absence of the target based upon the level of detectable signal measured at the defined temperature, wherein a detectable signal at the defined temperature is indicative of the presence of the target in the sample.

Embodiment 2. The method of Embodiment 1 wherein the enzyme is capable of digesting the sensor region of the first oligonucleotide component only when the target is present in the sample, and wherein step (b) comprises treating the mixture under conditions suitable for the enzyme to digest the sensor region of the first oligonucleotide component to thereby generate a first fragment comprising the first capture region and a second fragment connected to the first detection moiety.

Embodiment 3. The method of Embodiment 1 or Embodiment 2 wherein the method comprises:

(i) measuring a level of background signal or detectable signal generated by the first and second detection moieties in the mixture at the defined temperature

- at a timepoint prior to or during said treating the mixture, and
- at one or more subsequent timepoint(s) during or following said treating the mixture; and

(ii) determining a presence of or a change in the level of detectable signal which differs from the background signal and is indicative of the presence of the target in the sample.

Embodiment 4. The method of Embodiment 3 wherein step (c) comprises measuring the detectable signal and/or any said background signal:

- at one or more timepoints prior to said treating;
- at one or more timepoints during said treating;
- at one or more timepoints after said treating;
- at one or more timepoints during said treating and at one or more timepoints after said treating;
- at one or more timepoints prior to said treating and at one or more timepoints after said treating; or
- at one or more timepoints before and during said treating and at one or more timepoints after said treating.

Embodiment 5. The method of Embodiment 3 or Embodiment 4 wherein step (d) comprises using a predetermined threshold value to determine if the detectable signal differs from any said background signal at the defined temperature.

Embodiment 6. The method of Embodiment 1 or Embodiment 2 further comprising measuring a level of control background signal generated at the defined temperature in a control mix,

and wherein step (c) comprises measuring a level of the background or detectable signal in the mixture contacted by the sample or derivative thereof,

and wherein step (d) comprises determining whether a detectable signal that differs from the control background signal is generated and indicative of the presence of the target in the sample.

Embodiment 7. The method of Embodiment 1 or Embodiment 2 further comprising:

measuring a level of control background signal generated at the defined temperature in a control mix, and

determining whether the level of control background signal measured in the control mix differs from the level of background signal or detectable signal measured in the mixture at step (c),

wherein a difference in the level of background signal or detectable signal measured in the mixture at step (c) compared to the level of control background signal measured in the control mix is indicative of the presence of the target in the sample.

Embodiment 8. The method of Embodiment 6 or Embodiment 7 wherein the control mix does not comprise the target but is otherwise equivalent to the mixture.

Embodiment 9. The method of Embodiment 6 or Embodiment 7 wherein the control mix does not comprise the enzyme but is otherwise equivalent to the mixture.

Embodiment 10. The method of Embodiment 1 or Embodiment 2 further comprising:

measuring a level of control detectable signal generated at the defined temperature in a control mix, wherein the control mix comprises a predetermined amount of the target but is otherwise equivalent to the mixture; and

determining whether the level of control detectable signal measured in the control mix differs from the level of background signal or detectable signal measured in the mixture at step (c),

wherein a difference in the level of background signal or detectable signal measured in the mixture at step (c) compared to the level of control detectable signal measured in the control mix is indicative of the presence and/or amount of the target in the sample.

Embodiment 11. The method of any one of Embodiments 1 to 10 wherein the target is a nucleic acid and at least a portion of the sensor region hybridises to a complementary sequence in the target to thereby form a duplex between the sensor region and the target.

Embodiment 12. The method of Embodiment 11 wherein the enzyme is an endonuclease that recognises a sequence in the duplex.

Embodiment 13. The method of Embodiment 12 wherein the endonuclease digests at least one strand of the duplex to thereby form the first and second fragments.

Embodiment 14. The method of Embodiment 12 wherein the endonuclease is a nicking endonuclease that digests the sensor region of the first oligonucleotide component after formation of the duplex to thereby form the first and second fragments.

Embodiment 15. The method of Embodiment 11 wherein the enzyme is an exonuclease that hydrolyses the sensor region of the first oligonucleotide component after formation of the duplex to thereby form the first and second fragments.

Embodiment 16. The method of Embodiment 15 wherein the exonuclease is a polymerase with exonuclease activity.

Embodiment 17. The method of Embodiment 16 wherein

- the target is a nucleic acid,
- at least a portion of the sensor region hybridises to a complementary sequence in the target to thereby form a duplex between the sensor region and the target,
- said mixture further comprises a target primer capable of binding to the target at a region upstream of said complementary sequence,
- said treating the mixture comprises:
 - hybridisation of the target primer to the target by complementary base pairing,
 - extending the primer using the polymerase with exonuclease activity and using the nucleic acid target as a template,
 - wherein the polymerase comprising exonuclease activity digests the sensor region of the first oligonucleotide component after formation of the duplex to thereby form the first and second fragments.

Embodiment 18. The method of any one of Embodiments 1 to 10 wherein the enzyme is a DNAzyme.

Embodiment 19. The method of any one of Embodiments 1 to 10 wherein the target is a nucleic acid and the sensor region of the first oligonucleotide component is not complementary to the target.

Embodiment 20. The method of any one of Embodiments 1 to 10 or 19 wherein the target is a nucleic acid and the enzyme is a multi-component nucleic acid enzyme (MNAzyme) comprising two partzyme oligonucleotides capable of self-assembling to form the MNAzyme only in the presence of the target.

Embodiment 21. The method of Embodiment 20 wherein said treating comprises:

hybridising sensor arms of the MNAzyme to the target by complementary base pairing, and

hybridising substrate arms of the MNAzyme to at least a portion of the sensor region of the first oligonucleotide component by complementary base pairing to facilitate cleavage of the first oligonucleotide component and generation of the first and second fragments.

Embodiment 22. The method of any one of Embodiments 1 to 21 wherein the target is a nucleic acid.

Embodiment 23. The method of Embodiment 22 wherein the target is an amplicon of a nucleic acid.

Embodiment 24. The method of Embodiment 23 wherein the amplicon is produced by an amplification reaction selected from the group consisting of polymerase chain reaction (PCR), strand displacement amplification (SDA), nicking enzyme amplification reaction (NEAR), helicase dependent amplification (HDA), Recombinase Polymerase Amplification (RPA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), transcription-mediated amplification (TMA), self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), Ligase Chain Reaction (LCR) or Ramification Amplification Method (RAM) and reverse transcription polymerase chain reaction (RT-PCR).

Embodiment 25. The method of Embodiment 24, wherein said detecting:

- occurs prior to said amplification or within 1, 2, 3, 4, or 5 cycles of said amplification commencing; and/or
- occurs after completion of said amplification.

Embodiment 26. The method of any one of Embodiments 23 to 25 wherein said determining the presence or absence of the target comprises a melt curve analysis.

Embodiment 27. The method of any one of Embodiments 1 to 10 wherein:

- the enzyme is a DNAzyme or a ribozyme requiring a co-factor for catalytic activity,
 - said treating of the mixture comprises using conditions suitable for:
 - binding of the cofactor to the DNAzyme or ribozyme to render it catalytically active,
 - hybridisation of the DNAzyme or ribozyme to the first oligonucleotide component by complementary base pairing,
 - catalytic activity of the DNAzyme or ribozyme to thereby digest the first oligonucleotide component and generate the first fragment and the second fragment, and
 - the target is the co-factor.

Embodiment 28. The method of Embodiment 27 wherein the co-factor is a metal ion, such as a metal ion selected from: Mg^{2+} , Mn^{2+} , Ca^{2+} and Pb^{2+} .

Embodiment 29. The method of any one of Embodiments 1 to 10 wherein the enzyme is an aptazyme wherein:

- the sensor region comprises a substrate for an aptazyme;
- the target is an analyte, protein, peptide, compound or nucleic acid;
- the mixture comprises an aptazyme comprising an aptamer capable of binding to the target; and
 - said treating the mixture further comprises binding of the aptazyme to the target and to the sensor region to facilitate cleavage of the first oligonucleotide component to thereby generate the first fragment and the second fragment.

Embodiment 30. A method for determining the presence or absence of a target in a sample, the method comprising:

(a) preparing a mixture for a reaction by contacting the sample or a derivative thereof putatively comprising the target with:

- a multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,

wherein the first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a first double-stranded portion,

wherein the first oligonucleotide component further comprises a sensor region capable of serving as a substrate for an enzyme,

wherein the first oligonucleotide component is connected to a first detection moiety and the second oligonucleotide component is connected to a second detection moiety;

- an enzyme capable of modifying the sensor region of the first oligonucleotide component only when the target is present in the sample;

(b) treating the mixture under conditions suitable for the enzyme to modify the sensor region of the first oligonucleotide component to thereby generate a first fragment comprising the first capture region and a second fragment connected to the first detection moiety, thereby enabling the first and second detection moieties to spatially separate and generate a first detectable signal,

(c) measuring a level of detectable signal generated at a plurality of temperatures including at temperatures below and above a temperature at which the first capture region hybridises to the second oligonucleotide component; and

(d) determining the presence or absence of the target based upon the presence or absence of a change in detectable signal generating a melt curve peak, wherein absence of a melt curve peak is indicative of the presence of the target in the sample and presence of a melt curve peak is indicative of the absence of the target in the sample.

Embodiment 31. The method of any one of Embodiments 1 to 30 wherein the second oligonucleotide component is directly labelled with the second detection moiety.

Embodiment 32. The method of any one of Embodiments 1 to 31 wherein the first fragment is not directly labelled with a detection moiety.

Embodiment 33. The method of any one of Embodiments 1 to 32 wherein the second fragment is not directly labelled with the first detection moiety.

Embodiment 34. The method of any one of Embodiments 1 to 33 wherein the first oligonucleotide component is not directly labelled with the first detection moiety.

Embodiment 35. The method of any one of Embodiments 1 to 34 wherein the first oligonucleotide component is connected to the first detection moiety via a third oligonucleotide component, wherein

the first oligonucleotide component further comprises a second capture region capable of hybridisation to the third oligonucleotide component by complementary base pairing to form a second double-stranded portion, and

the third oligonucleotide component is directly labelled with the first detection moiety.

Embodiment 36. The method of Embodiment 35 wherein the first double-stranded portion and the second double-stranded portion of the multi-component temperature-controlled probe have a melting temperature (T_m) that is above the defined temperature.

Embodiment 37. The method of Embodiment 35 or Embodiment 36 wherein the T_m of the first double-stranded portion is less than the T_m of the second double-stranded portion.

Embodiment 38. The method of Embodiment 35 or Embodiment 36 wherein the T_m of the second double-stranded portion is less than the T_m of the first double-stranded portion.

Embodiment 39. The method of any one of Embodiments 1 to 31 wherein the first oligonucleotide component is directly labelled with the first detection moiety.

Embodiment 40. The method of any one of Embodiments 1 to 39 wherein the multi-component temperature-controlled probe does not comprise more than two detection moieties.

Embodiment 41. The method of any one of Embodiments 1 to 40 wherein the first oligonucleotide component is not directly labelled with more than one detection moiety.

Embodiment 42. The method of any one of Embodiments 1 to 41 wherein the first oligonucleotide component is not connected to more than one detection moiety.

Embodiment 43. The method of any one of Embodiments 1 to 42 wherein the second oligonucleotide component is not directly labelled with more than one detection moiety.

Embodiment 44. The method of any one of Embodiments 1 to 43 wherein the second oligonucleotide component is not connected to more than one detection moiety.

Embodiment 45. The method of any one of Embodiments 1 to 44 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore.

Embodiment 46. The method of any one of Embodiments 1 to 45 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore;

and wherein the multi-component temperature-controlled probe does not comprise more than one quencher.

Embodiment 47. The method of any one of Embodiments 1 to 46 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore;

and wherein the detectable signal is fluorescence emitted in the presence of the target.

Embodiment 48. The method of any one of Embodiments 1 to 47 wherein neither the first oligonucleotide component, the second oligonucleotide component nor the third oligonucleotide component serve as a primer for a DNA polymerase in an extension reaction.

Embodiment 49. The method of any one of Embodiments 1 to 48 wherein neither the first oligonucleotide component, the second oligonucleotide component nor the third oligonucleotide component serve as a template for a DNA polymerase in an extension reaction.

Embodiment 50. The method of any one of Embodiments 1 to 49 wherein neither the second oligonucleotide component nor the third oligonucleotide component is enzymatically cleaved or degraded.

Embodiment 51. The method of any one of Embodiments 1 to 50 wherein the sensor region is located between the first capture region and the first detection moiety.

Embodiment 52. The method of any one of Embodiments 1 to 51 wherein following said treating the mixture the first fragment is capable of hybridizing to the second oligonucleotide component via the first capture region.

Embodiment 53. The method of any one of Embodiments 1 to 52 wherein the biological sample is obtained from a subject.

Embodiment 54. The method of any one of Embodiments 1 to 53 wherein generation of the detectable signal at the defined temperature is not reversible.

Embodiment 55. The method of any one of Embodiments 1 to 54 wherein the method is performed *in vitro*.

Embodiment 56. The method of any one of Embodiments 1 to 55 wherein the method is performed *ex vivo*.

Embodiment 57. A method for determining the presence or absence of a first target and a second target in a sample, the method comprising:

(a) preparing a mixture for a reaction by contacting the sample or a derivative thereof putatively comprising the first and/or second target with:

- a multi-component temperature-controlled probe for detection of the first target, the multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,

wherein the first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a first double-stranded portion,

wherein the first oligonucleotide component further comprises a sensor region capable of serving as a substrate for an enzyme,

wherein the first oligonucleotide component is connected to a first detection moiety and the second oligonucleotide component is connected to a second detection moiety,

- a second nucleic acid probe for detection of the second target, the second nucleic acid probe comprising third and fourth detection moieties,

wherein the first and second detection moieties are capable of generating a first detectable signal, and the third and fourth detection moieties are capable of generating a second detectable signal, and

- a first enzyme capable of modifying the sensor region of the first oligonucleotide component only when the first target is present in the sample;

(b) treating the mixture under conditions suitable for:

- the first enzyme to modify the sensor region of the first oligonucleotide component to thereby generate a first fragment comprising the first capture region and a second fragment connected to the first detection moiety, thereby enabling the first and second detection moieties to spatially separate and generate a first detectable signal,

- the second target to induce a modification of the second nucleic acid probe, thereby enabling the third and fourth detection moieties to spatially separate and generate a second detectable signal;

(c) measuring a level of background or detectable signal:

- at a first temperature at or below which the first capture region is hybridised to the second oligonucleotide component,

- at a second temperature at or above which the first capture region is not hybridised to the second oligonucleotide component,

- (d) determining whether at one or more timepoints during or after said treating:

- a first detectable signal is generated at the first temperature at or below which the first capture region is hybridised to the second oligonucleotide component,

- a second detectable signal arising from said modification of the second nucleic acid probe is generated at the second temperature, wherein the second detectable signal is indicative of the presence of the second target in the sample.

Embodiment 58. The method of Embodiment 57 wherein the first enzyme is capable of digesting the sensor region of the first oligonucleotide component only when the first target is present in the sample, and wherein step (b) comprises treating the mixture under conditions suitable for the first enzyme to digest the sensor region of the first oligonucleotide component to thereby generate a first fragment comprising the first capture region and a second fragment connected to the first detection moiety.

Embodiment 59. The method of Embodiment 57 or Embodiment 58 wherein a first detectable signal at the first temperature is indicative of the presence of the first target in the sample.

Embodiment 60. The method of Embodiment 59 wherein the presence of the first target is determined at the first temperature based upon the first detectable signal generated at the first temperature.

Embodiment 61. The method of any one of Embodiments 57 to 60 wherein the presence of the second target is determined at the second temperature based upon the second detectable signal generated at the second temperature.

Embodiment 62. The method of Embodiment 57 or Embodiment 58 wherein:

- (i) at the first temperature

- a first detectable signal is generated in the presence of the first target,

- a second detectable signal is generated in the presence of the second target, or

- a first detectable signal and a second detectable signal is generated in the presence of both the first target and the second target; and

(ii) a second detectable signal is generated at the second temperature only in the presence of the second target.

Embodiment 63. The method of Embodiment 62 wherein the presence of the first target is determined by subtracting any second detectable signal detected at the second temperature from any first and/or second detectable signal detected at the first temperature.

Embodiment 64. The method of any one of Embodiments 57 to 63 wherein the method comprises:

- measuring a level of background signal or detectable signal at the first and second temperatures generated by the first and second detection moieties and by the third and fourth detection moieties in the mixture,
- determining a presence of or a change in the level of the first detectable signal which differs from the background signal and is indicative of the presence of the first target in the sample, and
- determining a presence of or a change in the level of the second detectable signal arising from said modification generated at the second temperature which differs from the background signal and is indicative of the presence of the second target in the sample.

Embodiment 65. The method of any one of Embodiments 57 to 64 wherein at the second temperature, dissociation of the second oligonucleotide component from the capture region of either the first oligonucleotide component present in the absence of the first target, or the first fragment generated by modification of the first oligonucleotide component in the presence of the first target generate an equal, similar or equivalent background signal.

Embodiment 66. The method of any one of Embodiments 57 to 65 wherein said determining comprises detection of the first detectable signal and/or any said background signal:

- at one or more timepoints prior to said treating
- at one or more timepoints during said treating;
- at one or more timepoints after said treating;

- at one or more timepoints during said treating and at one or more timepoints after said treating;
- at one or more timepoints prior to said treating and at one or more timepoints after said treating; or
- at one or more timepoints before and during said treating and at one or more timepoints after said treating.

Embodiment 67. The method of any one of Embodiments 57 to 66, wherein said determining in part (d) comprises:

- using a predetermined threshold value to determine if the first detectable signal differs from any said background signal at the first temperature; and/or
- using a predetermined threshold value to determine if the second detectable signal differs from any said background signal at the second temperature.

Embodiment 68. The method of any one of Embodiments 57 to 63 comprising:

measuring a level of first control background signal at the first temperature provided by the first and second detection moieties and by the third and fourth detection moieties in a control mix;

measuring a level of second control background signal at the second temperature provided by the first and second detection moieties and by the third and fourth detection moieties in the control mix;

determining whether a level of the first detectable signal generated at the first temperature at step (c) in the mixture contacted by the sample or derivative thereof differs from the level of first control background signal measured in the control mix, wherein a difference in the level of detectable signal measured in the mixture at the first temperature at step (c) compared to the first control background signal measured in the control mix is indicative of the first target in the sample; and

determining whether a level of the second detectable signal generated at the second temperature at step (c) in the mixture contacted by the sample or derivative thereof differs from the level of second control background signal measured in the control mix, wherein a difference in the level of detectable signal measured in the mixture at the second temperature at step (c) compared to the second control background signal measured in the control mix is indicative of the second target in the sample.

Embodiment 69. The method of Embodiment 68 wherein the control mix does not comprise:

- the first target;
- the second target; or
- the first and second targets,

but is otherwise equivalent to the mixture.

Embodiment 70. The method of Embodiment 68 wherein the control mix does not comprise the first enzyme but is otherwise equivalent to the mixture.

Embodiment 71. The method of any one of Embodiments 57 to 63 further comprising:

measuring a level of first control detectable signal generated at the first temperature in a control mix, wherein the control mix comprises a predetermined amount of

- the first target,
- the second target, or
- the first and second targets,

but is otherwise equivalent to the mixture;

measuring a level of second control detectable signal generated at the second temperature in the control mix;

determining whether a level of the first detectable signal generated at the first temperature at step (c) in the mixture contacted by the sample or derivative thereof differs from the level of first control detectable signal measured in the control mix, wherein a difference in the level of detectable signal measured in the mixture at the first temperature at step (c) compared to the first control detectable signal measured in the control mix is indicative of the first target in the sample; and

determining whether a level of the second detectable signal generated at the second temperature at step (c) in the mixture contacted by the sample or derivative thereof differs from the level of second control detectable signal measured in the control mix, wherein a difference in the level of detectable signal measured in the mixture at the second temperature at step (c) compared to the second control detectable signal measured in the control mix is indicative of the second target in the sample.

Embodiment 72. The method of any one of Embodiments 57 to 67 wherein part (c) comprises measuring a first background signal at or within 1°C, 2°C, 3°C, 4°C or 5°C of

the first temperature, and a second background signal at or within 1°C, 2°C, 3°C, 4°C or 5°C of the second temperature.

Embodiment 73. The method of Embodiment 72 wherein part (d) comprises determining whether at one or more time points during or after said treating:

a first detectable signal is generated at the first temperature which differs from the first background signal and is indicative of the presence of the first target in the sample; and

a second detectable signal is generated at the second temperature which differs from the second background signal and is indicative of the presence of the second target in the sample.

Embodiment 74. The method of any one of Embodiments 57 to 73 wherein at the first temperature the third and fourth detection moieties do not generate a signal which differs from the background signal.

Embodiment 75. The method of any one of Embodiments 57 to 74 wherein at the second temperature the first and second detection moieties do not generate a signal which differs from the background signal.

Embodiment 76. The method of any one of Embodiments 57 to 75 wherein the first and second detectable signals are detectable by a single detector.

Embodiment 77. The method of any one of Embodiments 57 to 76 wherein the first and second detectable signals are detectable in the same fluorescent channel.

Embodiment 78. The method of any one of Embodiments 57 to 77 wherein the first and second detectable signals are detectable as fluorescent emission at a single wavelength.

Embodiment 79. The method of any one of Embodiments 57 to 78, wherein the first and second detection moieties, and the third and fourth detection moieties emit a detectable signal at the same or similar wavelength which can be detected in the same fluorescence channel.

Embodiment 80. The method of any one of Embodiments 57 to 79 wherein the second oligonucleotide component is directly labelled with the second detection moiety.

Embodiment 81. The method of any one of Embodiments 57 to 80 wherein the first fragment is not directly labelled with a detection moiety.

Embodiment 82. The method of any one of Embodiments 57 to 81 wherein the second fragment is not directly labelled with the first detection moiety.

Embodiment 83. The method of any one of Embodiments 57 to 82 wherein the first oligonucleotide component is not directly labelled with the first detection moiety.

Embodiment 84. The method of any one of Embodiments 57 to 83 wherein the first oligonucleotide component is connected to the first detection moiety via a third oligonucleotide component, wherein

the first oligonucleotide component further comprises a second capture region capable of hybridisation to the third oligonucleotide component by complementary base pairing to form a second double-stranded portion, and

the third oligonucleotide component is directly labelled with the first detection moiety.

Embodiment 85. The method of Embodiment 84 wherein the first double-stranded portion and the second double-stranded portion of the multi-component temperature-controlled probe have a T_m that is above the first temperature.

Embodiment 86. The method of Embodiment 84 or Embodiment 85 wherein the first double-stranded portion and/or the second double-stranded portion of the multi-component temperature-controlled probe have a T_m that is below the second temperature.

Embodiment 87. The method of any one of Embodiments 84 to 86 wherein the T_m of the first double-stranded portion is less than the T_m of the second double-stranded portion.

Embodiment 88. The method of any one of Embodiments 84 to 86 wherein the T_m of the second double-stranded portion is less than the T_m of the first double-stranded portion.

Embodiment 89. The method of any one of Embodiments 57 to 80 wherein the first oligonucleotide component is directly labelled with the first detection moiety.

Embodiment 90. The method of any one of Embodiments 57 to 89 wherein the multi-component temperature-controlled probe does not comprise more than two detection moieties.

Embodiment 91. The method of any one of Embodiments 57 to 90 wherein the first oligonucleotide component is not directly labelled with more than one detection moiety.

Embodiment 92. The method of any one of Embodiments 57 to 91 wherein the first oligonucleotide component is not connected to more than one detection moiety.

Embodiment 93. The method of any one of Embodiments 57 to 92 wherein the second oligonucleotide component is not directly labelled with more than one detection moiety.

Embodiment 94. The method of any one of Embodiments 57 to 93 wherein the second oligonucleotide component is not connected to more than one detection moiety.

Embodiment 95. The method of any one of Embodiments 57 to 94 wherein the second nucleic acid probe is directly labelled with the third and fourth detection moieties.

Embodiment 96. The method of any one of Embodiments 57 to 94 wherein the second nucleic acid probe is not directly labelled with the third and fourth detection moieties.

Embodiment 97. The method of any one of Embodiments 57 to 96 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore.

Embodiment 98. The method of any one of Embodiments 57 to 97 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore;

and wherein the multi-component temperature-controlled probe does not comprise more than one quencher.

Embodiment 99. The method of any one of Embodiment 57 to 98 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore;

and wherein the first detectable signal is fluorescence emitted in the presence of the first target.

Embodiment 100. The method of any one of Embodiments 57 to 99 wherein neither the first oligonucleotide component, the second oligonucleotide component nor the third oligonucleotide component serve as a primer for a DNA polymerase in an extension reaction.

Embodiment 101. The method of any one of Embodiments 57 to 100 wherein neither the first oligonucleotide component, the second oligonucleotide component nor the third oligonucleotide component serve as a template for a DNA polymerase in an extension reaction.

Embodiment 102. The method of any one of Embodiments 57 to 101 wherein neither the second oligonucleotide component nor the third oligonucleotide component is enzymatically cleaved or degraded.

Embodiment 103. The method of any one of Embodiments 57 to 102 wherein the sensor region is located between the first capture region and the first detection moiety.

Embodiment 104. The method of any one of Embodiments 57 to 103 wherein following said treating the first fragment is capable of hybridizing to the second oligonucleotide component via the first capture region.

Embodiment 105. The method of any one of Embodiments 57 to 104 wherein:

the third detection moiety is a fluorophore, and the fourth detection moiety is a quencher; or

the third detection moiety is a quencher, and the fourth detection moiety is a fluorophore.

Embodiment 106. The method of any one of Embodiments 57 to 105 wherein the first target is a nucleic acid and at least a portion of the sensor region hybridises to a complementary sequence in the first target to thereby form a duplex between the sensor region and the first target.

Embodiment 107. The method of Embodiment 106 wherein the first enzyme is an endonuclease that recognises a sequence in the duplex.

Embodiment 108. The method of Embodiment 107 wherein the endonuclease digests the duplex to thereby form the first and second fragments.

Embodiment 109. The method of Embodiment 107 wherein the endonuclease is a nicking endonuclease that digests the sensor region of the first oligonucleotide component after formation of the duplex to thereby form the first and second fragments.

Embodiment 110. The method of Embodiment 106 wherein the first enzyme is an exonuclease that hydrolyses the sensor region of the first oligonucleotide component after formation of the duplex to thereby form the first and second fragments.

Embodiment 111. The method of Embodiment 110 wherein the exonuclease is a polymerase with exonuclease activity.

Embodiment 112. The method of Embodiment 111 wherein

- the first target is a nucleic acid,

- at least a portion of the sensor region hybridises to a complementary sequence in the first target to thereby form a duplex between the sensor region and the first target,
- said mixture further comprises a first target primer capable of binding to the first target at a region upstream of said complementary sequence,
- said treating the mixture comprises:
 - hybridisation of the first target primer to the first target by complementary base pairing,
 - extending the primer using the polymerase with exonuclease activity and using the first target as a template,

wherein the polymerase comprising exonuclease activity digests the sensor region of the first oligonucleotide component after formation of the duplex.

Embodiment 113. The method of any one of Embodiments 57 to 105 wherein the first enzyme is a DNAzyme.

Embodiment 114. The method of any one of Embodiments 57 to 105 wherein the first target is a nucleic acid and the sensor region of the first oligonucleotide component is not complementary to the first target.

Embodiment 115. The method of any one of Embodiments 57 to 105 or 114 wherein the first target is a nucleic acid and the first enzyme is a first target multi-component nucleic acid enzyme (MNAzyme) comprising two partzyme oligonucleotides capable of self-assembling to form the first target MNAzyme only in the presence of the first target.

Embodiment 116. The method of Embodiment 115 wherein said treating comprises:

- hybridising sensor arms of the first target MNAzyme to the first target by complementary base pairing, and
- hybridising substrate arms of the first target MNAzyme to at least a portion of the sensor region of the first oligonucleotide component by complementary base pairing to facilitate cleavage of the first oligonucleotide component and generation of the first and second fragments.

Embodiment 117. The method of any one of Embodiments 57 to 116 wherein:

- the second nucleic acid probe is a substrate for a second target multi-component nucleic acid enzyme (MNAzyme) the second target MNAzyme comprising two partzyme oligonucleotides capable of self-assembling to form the second target MNAzyme only in the presence of the second target;

- the mixture further comprises:

- the second target MNAzyme is capable of cleaving the second nucleic acid probe only when the second target is present in the sample;

- said treating further comprises:

- hybridising sensor arms of the second target MNAzyme to the second target by complementary base pairing, and

- hybridising substrate arms of the second target MNAzyme to the second nucleic acid probe by complementary base pairing to facilitate cleavage of the second nucleic acid probe thereby providing said modification to the second nucleic acid probe and enabling the third and fourth detection moieties to spatially separate and generate the second detectable signal.

Embodiment 118. The method of Embodiment 117 wherein the second nucleic acid probe is a stem-loop oligonucleotide comprising a double-stranded stem portion of hybridised nucleotides opposing strands of which are linked by an unbroken single-stranded loop portion of unhybridised nucleotides of which all or a portion is complementary to the substrate arms of the second target MNAzyme.

Embodiment 119. The method of Embodiment 118 wherein the stem-loop oligonucleotide is an intact stem-loop oligonucleotide and the said modification comprises cleavage of the loop portion and the formation of a split stem-loop oligonucleotide.

Embodiment 120. The method of any one of Embodiments 57 to 116 wherein:

- the second target is a nucleic acid,

- the second nucleic acid probe is a stem-loop oligonucleotide comprising a double-stranded stem portion of hybridised nucleotides opposing strands of which are linked by an unbroken single-stranded loop portion of unhybridised nucleotides of which all or a portion is complementary to the second target,

- the mixture further comprises a polymerase with exonuclease activity,

- said treating the mixture comprises using conditions suitable for:
 - hybridisation of the second target to the single-stranded loop portion of the stem-loop oligonucleotide by complementary base pairing to form a first double-stranded sequence comprising a portion of the second target,
 - hybridisation of a primer to the second target to form a second double-stranded sequence located upstream relative to the first double-stranded sequence comprising the portion of the second target,
 - extending the primer using the polymerase with exonuclease activity and using the second target as a template,
 - wherein the polymerase comprising exonuclease activity digests the single-stranded loop portion of the first double-stranded sequence and thereby forms a split stem-loop oligonucleotide.

Embodiment 121. The method of any one of Embodiments 57 to 116, wherein:

- the second target is a nucleic acid,
- the second nucleic acid probe is a stem-loop oligonucleotide comprising a double-stranded stem portion of hybridised nucleotides opposing strands of which are linked by an unbroken single-stranded loop portion of unhybridised nucleotides of which all or a portion is complementary to the second target,
- the mixture further comprises an endonuclease, and
- said treating the mixture comprises using conditions suitable for:
 - hybridisation of the second target to the single-stranded loop portion of the stem-loop oligonucleotide by complementary base pairing to form a double-stranded sequence comprising a portion of the second target,
 - association of the endonuclease with the double-stranded sequence comprising a portion of the second target, and
 - catalytic activity of endonuclease allowing it to digest the single-stranded loop portion of the double-stranded sequence and thereby form a split stem-loop oligonucleotide.

Embodiment 122. The method of any one of Embodiments 118 to 121 wherein:

- the stem portion of the intact stem-loop oligonucleotide has a melting temperature (T_m) that is above the T_m of the stem portion of the split stem-loop oligonucleotide;

- the first temperature is below the T_m of the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is below the T_m of the stem portion of the intact stem-loop oligonucleotide, and is above the T_m of the stem portion of the split stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

Embodiment 123. The method of any one of Embodiments 118 to 122 wherein the T_m of the stem portion of the split stem-loop oligonucleotide is above the first temperature.

Embodiment 124. The method of any one of Embodiments 118 to 123 wherein the T_m of the stem portion of the intact and split stem-loop oligonucleotide(s) is above the T_m of the first double-stranded portion of the multi-component temperature-controlled probe.

Embodiment 125. The method of any one of Embodiments 57 to 116 wherein:

the second nucleic acid probe is a stem-loop oligonucleotide comprising a double-stranded stem portion of hybridised nucleotides opposing strands of which are linked by an unbroken single-stranded loop portion of unhybridized nucleotides of which all or a portion is complementary to the second target, and wherein

the modification of the second nucleic acid probe is a conformational change arising from hybridisation of the second target to the single-stranded loop portion by complementary base pairing that causes spatial separation of the third and fourth detection moieties.

Embodiment 126. The method of Embodiment 125 wherein the conformational change is dissociation of the opposing strands in the double-stranded stem portion of the second nucleic acid probe.

Embodiment 127. The method of any one of Embodiments 118 to 126 wherein the third and fourth detection moieties are connected to opposing strands of the double-stranded stem portion of the second nucleic acid probe.

Embodiment 128. The method of any one of Embodiments 57 to 116, wherein:

- the second target is a nucleic acid,

- the mixture further comprises:
 - a primer complementary to a first sequence in the second target,
 - a pitcher oligonucleotide comprising a region complementary to a second sequence in the second target that differs from the first sequence, and a tag portion that is not complementary to the second target,
 - a first polymerase comprising exonuclease activity, and
 - optionally a second polymerase, and
- said treating the mixture comprises:
 - suitable conditions to hybridise the primer and the pitcher oligonucleotide to the second target,
 - extending the primer using the first or second polymerase and the second target as a template to thereby cleave off the tag portion,
 - hybridising the cleaved tag portion to the second nucleic acid probe by complementary base pairing,
 - and extending the tag portion using the polymerase and the second nucleic acid probe as a template to generate a double-stranded catcher sequence comprising the second nucleic acid probe thereby providing said modification to the second nucleic acid probe and enabling the third and fourth detection moieties to provide the second detectable signal.

Embodiment 129. The method of Embodiment 128 wherein:

- the double-stranded catcher sequence has a T_m that is above the first temperature; and
- the second temperature is below the T_m of the double-stranded catcher sequence.

Embodiment 130. The method of Embodiment 128 or Embodiment 129 wherein said extending the tag portion spatially separates the third and fourth detection moieties to thereby generate the second detectable signal.

Embodiment 131. The method of any one of Embodiments 57 to 116 wherein the second target is a nucleic acid and the second nucleic acid probe is a two-part probe comprising a first part oligonucleotide and a second part oligonucleotide, wherein:

- the first part oligonucleotide is complementary to a first portion of the second target,

- the second part oligonucleotide is complementary to a second portion of the second target,
- the first and second portions of the second nucleic acid target flank one another but do not overlap,
- said treating the mixture comprises:
 - forming a duplex structure comprising:
 - a first double-stranded portion by hybridising the first part oligonucleotide to the second target by complementary base pairing, and
 - a second double-stranded portion by hybridising the second part oligonucleotide to the second target by complementary base pairing,
 - thereby bringing the first and second part oligonucleotides into proximity and providing said modification to the second nucleic acid probe enabling the third and fourth detection moieties to come into close proximity and generate the second detectable signal.

Embodiment 132. The method of Embodiment 131 wherein the second detectable signal is a decrease in fluorescence.

Embodiment 133. The method of Embodiment 131 wherein the second detectable signal is an increase in fluorescence.

Embodiment 134. The method of any one of Embodiments 57 to 116, wherein:

- the second target is a nucleic acid,
- the second nucleic acid probe comprises a sequence that is complementary to the second target,
- the mixture further comprises:
 - a primer complementary to a portion of the second target, and
 - a polymerase with exonuclease activity;
- said treating the mixture comprises:
 - hybridising the primer to the second target by complementary base pairing,
 - hybridising the second nucleic acid probe to the second target by complementary base pairing,
 - extending the primer using the polymerase and the second target as a template to thereby digest the second nucleic acid probe and provide said

modification to the second nucleic acid probe enabling the third and fourth detection moieties to spatially separate and generate the second detectable signal.

Embodiment 135. The method of any one of Embodiments 57 to 116, wherein:

- the second target is a nucleic acid,
- the mixture further comprises a restriction endonuclease capable of digesting a double-stranded duplex comprising the second target; and
- said treating the mixture comprises:
 - hybridising the second nucleic acid probe to the second target by complementary base pairing to thereby form the double-stranded duplex,
 - digesting the duplex using the restriction endonuclease to thereby provide said modification to the second nucleic acid probe and enabling the third and fourth detection moieties to spatially separate and generate the second detectable signal.

Embodiment 136. The method of any one of Embodiments 57 to 116 wherein:

- the second nucleic acid probe is a second multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,
 - wherein the first oligonucleotide component of the second multi-component temperature-controlled probe comprises a capture region capable of hybridisation to the second oligonucleotide component of the second multi-component temperature-controlled probe by complementary base pairing to form a double-stranded portion,
 - wherein the first oligonucleotide component of the second multi-component temperature-controlled probe further comprises a sensor region capable of serving as a substrate for a second enzyme only when the second target is present in the sample,
 - wherein the first oligonucleotide component of the second multi-component temperature-controlled probe is connected to the third detection moiety and the second oligonucleotide component of the second multi-component temperature-controlled probe is connected to the fourth detection moiety,
- the mixture further comprises the second enzyme,
- said treating of the mixture comprises:

suitable conditions for the second enzyme to digest the sensor region of the second multi-component temperature-controlled probe to thereby generate a first fragment comprising the capture region and a second fragment connected to the third detection moiety, thereby enabling the third and fourth detection moieties to spatially separate and generate a second detectable signal.

Embodiment 137. The method of Embodiment 136 wherein the first enzyme is the same as the second enzyme.

Embodiment 138. The method of Embodiment 136 or Embodiment 137 wherein the double-stranded portion or portions of the second multi-component temperature-controlled probe has a T_m above the T_m of the first double stranded portion and/or the second double stranded portion of the first multi-component temperature-controlled probe.

Embodiment 139. The method of any one of Embodiments 57 to 138 wherein:

- the first target is a nucleic acid;
- the second target is a nucleic acid; or
- the first target is a nucleic acid and the second target is a nucleic acid.

Embodiment 140. The method of Embodiment 139 wherein the first target and/or the second target is an amplicon of a nucleic acid.

Embodiment 141. The method of Embodiment 140 wherein the amplicon is produced by an amplification reaction selected from the group consisting of polymerase chain reaction (PCR), strand displacement amplification (SDA), nicking enzyme amplification reaction (NEAR), helicase dependent amplification (HDA), Recombinase Polymerase Amplification (RPA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), transcription-mediated amplification (TMA), self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), Ligase Chain Reaction (LCR) or Ramification Amplification Method (RAM) and reverse transcription polymerase chain reaction (RT-PCR).

Embodiment 142. The method of Embodiment 141 wherein said determining:

- occurs prior to said amplification or within 1, 2, 3, 4, or 5 cycles of said amplification commencing; and/or
- occurs after completion of said amplification.

Embodiment 143. The method of any one of Embodiments 140 to 142 wherein said determining the presence or absence of the first and second targets comprises a melt curve analysis.

Embodiment 144. The method of any one of Embodiments 57 to 116 or 136 to 143 wherein:

- the mixture further comprises a DNAzyme or a ribozyme requiring a co-factor for catalytic activity;
- said treating of the mixture comprises using conditions suitable for:
 - binding of the co-factor to the DNAzyme or ribozyme to render it catalytically active,
 - hybridisation of the DNAzyme or ribozyme to the second nucleic acid probe by complementary base pairing, and
 - catalytic activity of the DNAzyme or ribozyme to thereby digest the second nucleic acid probe and thereby provide said modification to the second nucleic acid probe enabling the third and fourth detection moieties to spatially separate and generate the second detectable signal, and
- the second target is the co-factor.

Embodiment 145. The method of any one of Embodiments 57 to 106 or 117 to 144 wherein:

- the first enzyme is a DNAzyme or a ribozyme requiring a co-factor for catalytic activity,
- said treating of the mixture comprises using conditions suitable for:
 - binding of the cofactor to the DNAzyme or ribozyme to render it catalytically active,
 - hybridisation of the DNAzyme or ribozyme to the first oligonucleotide component by complementary base pairing,

catalytic activity of the DNAzyme or ribozyme to thereby digest the first oligonucleotide component and generate the first fragment and the second fragment, and

- the first target is the co-factor.

Embodiment 146. The method of Embodiment 144 or Embodiment 145 wherein the co-factor is a metal ion, such as a metal ion selected from: Mg^{2+} , Mn^{2+} , Ca^{2+} and Pb^{2+} .

Embodiment 147. The method of any one of Embodiments 57 to 106 or 117 to 144 wherein the first enzyme is an aptazyme wherein:

- the sensor region comprises a substrate for an aptazyme;
- the first target is an analyte, protein, peptide, compound or nucleic acid;
- the mixture comprises an aptazyme comprising an aptamer capable of binding to the first target; and
- said treating the mixture further comprises binding of the aptazyme to the first target and to the sensor region to facilitate cleavage of the first oligonucleotide component to thereby generate the first fragment and the second fragment.

Embodiment 148. The method of any one of Embodiments 57 to 147 wherein generation of the first detectable signal is not reversible at the first temperature.

Embodiment 149. A method for determining the presence or absence of a first target and a second target in a sample, the method comprising:

(a) preparing a mixture for a reaction by contacting the sample or a derivative thereof putatively comprising the first and/or second target with:

- a multi-component temperature-controlled probe for detection of the first target, the multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,

wherein the first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a first double-stranded portion,

wherein the first oligonucleotide component further comprises a sensor region capable of serving as a substrate for an enzyme,

wherein the first oligonucleotide component is connected to a first detection moiety and the second oligonucleotide component is connected to a second detection moiety,

- a second nucleic acid probe for detection of the second target, the second nucleic acid probe comprising third and fourth detection moieties,

wherein the first and second detection moieties are capable of generating a first detectable signal, and the third and fourth detection moieties are capable of generating a second detectable signal, and

- a first enzyme capable of modifying the sensor region of the first oligonucleotide component only when the first target is present in the sample;

(b) treating the mixture under conditions suitable for:

- the first enzyme to modify the sensor region of the first oligonucleotide component to thereby generate a first fragment comprising the first capture region and a second fragment connected to the first detection moiety, thereby enabling the first and second detection moieties to spatially separate and generate a first detectable signal,

- the second target to induce a modification of the second nucleic acid probe, thereby enabling the third and fourth detection moieties to spatially separate and generate a second detectable signal;

(c) measuring a level of background or detectable signal:

- at a first temperature at or below which the first capture region is hybridised to the second oligonucleotide component,

- at a second temperature at or below which the first capture region is hybridised to the second oligonucleotide component,

(d) determining whether at one or more timepoints during or after said treating:

- a first detectable signal is generated at the second temperature at or below which the first capture region is hybridised to the second oligonucleotide component,

- a second detectable signal arising from said modification of the second nucleic acid probe is generated at the first temperature, wherein the second detectable signal is indicative of the presence of the second target in the sample.

Embodiment 150. The method of any one of Embodiments 57 to 149 wherein the first temperature is lower than the second temperature.

Embodiment 151. The method of any one of Embodiments 57 to 150 wherein the first enzyme does not digest the first target and/or the second target.

Embodiment 152. The method of any one of Embodiments 57 to 151 wherein the first temperature differs from the second temperature by more than: 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C or 60°C.

Embodiment 153. The method of any one of Embodiments 57 to 152 wherein the biological sample is obtained from a subject.

Embodiment 154. The method of any one of Embodiments 57 to 153 wherein the method is performed *in vitro*.

Embodiment 155. The method of any one of Embodiments 57 to 154 wherein the method is performed *ex vivo*.

Embodiment 156. A composition comprising:

- a multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,

- wherein the first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a first double-stranded portion,

- wherein the first oligonucleotide component is connected to a first detection moiety and the second oligonucleotide component is connected to a second detection moiety,

- wherein:

- the first oligonucleotide component further comprises a sensor region capable of serving as a substrate for an enzyme,

- wherein digestion of the sensor region by the enzyme generates a first fragment and a second fragment,

- and wherein the first fragment comprises the first capture region, and the second fragment is connected to the first detection moiety;

- and wherein:

the multi-component temperature-controlled probe does not comprise more than two detection moieties;

the first oligonucleotide component is not connected to more than one detection moiety; or

the first fragment is not directly labelled with a detection moiety.

Embodiment 157. The composition of Embodiment 156 wherein the first oligonucleotide component is hybridised to the second oligonucleotide component by complementary base pairing at the first capture region.

Embodiment 158. The composition of Embodiment 156 or Embodiment 157 wherein the second oligonucleotide component is not directly labelled with more than one detection moiety.

Embodiment 159. The composition of any one of Embodiments 156 to 158 wherein the second oligonucleotide component is not connected to more than one detection moiety.

Embodiment 160. The composition of any one of Embodiments 156 to 159 wherein the second oligonucleotide component is directly labelled with the second detection moiety.

Embodiment 161. The composition of any one of Embodiments 156 to 160 wherein the second fragment is not directly labelled with the first detection moiety.

Embodiment 162. The composition of any one of Embodiments 156 to 161 wherein the first oligonucleotide component is not directly labelled with the first detection moiety.

Embodiment 163. The composition of Embodiment 162 wherein the first oligonucleotide component is connected to the first detection moiety via a third oligonucleotide component, wherein

the first oligonucleotide component further comprises a second capture region capable of hybridisation to the third oligonucleotide component by complementary base pairing to form a second double-stranded portion, and

the third oligonucleotide component is directly labelled with the first detection moiety.

Embodiment 164. The composition of Embodiment 163 wherein the first capture region differs in length or sequence from the second capture region.

Embodiment 165. The composition of any one of Embodiments 156 to 160 wherein the first oligonucleotide component is directly labelled with the first detection moiety.

Embodiment 166. The composition of any one of Embodiments 156 to 165 wherein:
the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or
the first detection moiety is a quencher, and the second detection moiety is a fluorophore.

Embodiment 167. The composition of any one of Embodiments 156 to 166 wherein:
the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or
the first detection moiety is a quencher, and the second detection moiety is a fluorophore;
and wherein the multi-component temperature-controlled probe does not comprise more than one quencher.

Embodiment 168. The composition of any one of Embodiments 156 to 167 wherein the sensor region is located between the first capture region and the first detection moiety.

Embodiment 169. The composition of any one of Embodiments 156 to 168 wherein following digestion of the sensor region the first fragment is capable of hybridizing to the second oligonucleotide component via the first capture region.

Embodiment 170. The composition of any one of Embodiments 156 to 169, further comprising a multi-component nucleic acid enzyme (MNAzyme) comprising two partzyme oligonucleotides, each partzyme oligonucleotide having a substrate arm capable of hybridising to at least a portion of the sensor region of the first oligonucleotide component.

Embodiment 171. The composition of Embodiment 170 wherein the substrate arms of the two partzyme oligonucleotides are hybridised to the sensor region of the first oligonucleotide component.

Embodiment 172. The composition of any one of Embodiments 156 to 171, further comprising a DNAzyme capable of cleaving the sensor region of the first oligonucleotide component only in the presence of a target.

Embodiment 173. The composition of any one of Embodiments 156 to 172, further comprising an aptazyme capable of cleaving the sensor region of the first oligonucleotide component only in the presence of a target.

Embodiment 174. The composition of any one of Embodiments 156 to 173, further comprising a restriction endonuclease capable of cleaving the sensor region of the first oligonucleotide component only in the presence of a nucleic acid target.

Embodiment 175. The composition of any one of Embodiments 156 to 174, further comprising an exonuclease capable of digesting the sensor region of the first oligonucleotide component only in the presence of a nucleic acid target.

Embodiment 176. The composition of Embodiment 175 wherein the exonuclease is a polymerase with exonuclease activity.

Brief Description of the Drawings

Preferred embodiments of the present invention will now be described, by way of example only, with reference to the accompanying Figures as set out below.

Figure 1 Exemplary M-Tec Probes (i) An M-Tec-P suitable for cleavage by a PlexZyme (i.e., MNAzyme) assembled in the presence of a target. This M-Tec-P has two oligonucleotide components namely: a first oligonucleotide component (OC1) comprising, within its sensor region, a PlexZyme substrate, and a first detection moiety for example a quencher, at one terminus; and a second oligonucleotide component (OC2) labelled with a second detection moiety, for example a fluorophore, which is capable of hybridizing by complementary base pairing to the capture region of OC1. The position on OC1 which is amenable to enzymatic modification by the PlexZyme is located between

the first capture region and the region connected to the first detection moiety. In all figures the target is represented in a 3' to 5' orientation. In one embodiment, the OC2 hybridizes to a capture region of the OC1 which does not bind to the substrate binding arms of the PlexZyme. (ii) An M-Tec-H suitable for hydrolysis by exonuclease activity, for example, the 5'-3' exonuclease within the Taq polymerase in the presence of target. This M-Tec-H has two oligonucleotide components namely: a first oligonucleotide component OC1 comprising, within its sensor region, a target binding region, and a first detection moiety for example a quencher at one terminus; and a second oligonucleotide component OC2 labelled with a second detection moiety, for example a fluorophore, which is capable of hybridizing to the OC1 by complementary base pairing to the capture region of OC1. The region which is amenable to enzymatic modification is located on OC1 between the first capture region and the connected first detection moiety. In one embodiment, the OC2 hybridizes to a capture region of OC1 which does not bind to the target. In M-Tec Probes of either structure M-Tec-P or M-Tec-H, the positions of the first and second detection moieties, for example the fluorophore and the quencher, can be reversed as shown in (iii) and (iv) respectively. In some embodiments, the capture region may be located at or near the 5' terminus of OC1 and the first detection moiety is directly attached to or near the 3' terminus (LHS (i) – (iv)). In other embodiments, the capture region is located at or near the 3' terminus of OC1 and the first detection moiety is directly attached to or near the 5' terminus (RHS (i) – (iv)).

Figure 2 Exemplary components of M-Tec-P, PlexZymes and complexes formed in the presence of a target. (i) The components for an M-Tec-P may include an OC1 labelled with a quencher at one terminus and comprising a sensor region that serves as a substrate for a PlexZyme, and an OC2 labelled with a fluorophore which is capable of hybridizing to the OC1. (ii) The components for a PlexZyme may include two component PartZymes A and B, each of which has one region complementary to the target known as a target binding arm, a second region complementary to a PlexZyme substrate known as a substrate binding arm, and an intervening region which constitutes half of a catalytic core sequence. (iii) A target sequence for detection. (iv) A fully assembled PlexZyme bound to an M-Tec-P. When the PartZyme A and B target binding arms hybridize adjacently on a target a catalytic enzyme complex known as a PlexZyme forms. The sensor region of OC1 of the M-Tec-P can then hybridize to substrate binding arms of the PlexZyme. The PlexZyme is capable of cleaving multiple M-Tec-P substrates in a multiple turnover manner.

Figure 3 Exemplary structures formed by components of an M-Tec-P and fluorescence at various temperatures in the presence and absence of target. In all panels, uncleaved or cleaved OC1s are black and labelled with a quencher (Q); OC2s are grey and labelled with a fluorophore (F) which is either in a quenched state (circles) or an unquenched/fluorescent state (stars). PartZymes, unassembled or assembled into PlexZymes, are grey; target nucleic acids are white with a grey outline; Low temperatures are below the T_m for hybridization of the capture region of OC1 to OC2 (T_m OC1/OC2) whilst high temperatures are above the T_m OC1/OC2. Panel (i) illustrates reaction components at low temperatures in the presence of target. PartZymes assemble into PlexZymes which cleave the OC1 generating a first fragment and a second fragment and resulting in separation of the F and Q, which in turn results in an increase in fluorescence indicative of the presence of the target. At this temperature, the OC2 remains hybridized to the first fragment of the cleaved OC1. Panel (ii) illustrates reaction components at low temperatures in the absence of target. PartZymes are free in solution and uncleaved OC1s remain quenched due to close proximity of the F and Q since OC2 is still hybridized to OC1. Panel (iii) illustrates reaction components at high temperatures in the presence of target. PartZymes in general do not assemble into PlexZymes at high temperatures; however, when cleavage has previously occurred at the low temperature both cleaved and uncleaved OC1s may be present in the reaction mix but neither will be hybridized to the OC2. The contribution to background fluorescent signal is present due to separation of the F and Q. Panel (iv) illustrates reaction components at high temperatures in the absence of target. PartZymes are free in solution and uncleaved OC1s will not be hybridized to the OC2. The contribution to background fluorescent signal is present due to separation of the F and Q. During the course of a reaction, at low temperatures there will be an increase in fluorescence in the presence of target (i) but no increase in fluorescence in the absence of target (ii). At high temperatures, the level of fluorescence is equal regardless of the presence (iii) or absence (iv) of target. At high temperatures, the measured fluorescence arising from an M-Tec-P probe contributes to a background reading which does not change during the reaction.

Figure 4: Panels (i), (ii), (iii) and (iv) as previously described in Figure 3. The middle panels show exemplary amplification plots for PCR where fluorescence (y-axis) is plotted against PCR cycle number (x-axis). The top middle panel shows fluorescent signal obtained at low temperatures (below T_m OC1/OC2) in reactions containing high (A) or low (B) number of copies of target, or when no target (C) is present. The bottom middle

panel shows fluorescent signal obtained at high temperatures (above T_m OC1/OC2) in the same reactions.

Figure 5 illustrates three types of general designs for PlexZyme substrate probes which can be used alone or in conjunction with each other to facilitate the detection of target nucleic acids. All three probe types can be cleaved by PlexZymes in the presence of target resulting in an increase in fluorescence. Panel (i) illustrates a “standard” linear PlexZyme substrate which is dual labelled with a fluorophore (F) at one end and a quencher (Q) at the other. When linear probes are cleaved an increase in fluorescence above background can be observed across a broad temperature range (at low and high temperatures). Panel (ii) illustrates a hairpin stem-loop PlexZyme substrate (LOCS probe) which incorporates a PlexZyme substrate region in the loop, and a stem region labelled with a fluorophore (F) at one end and a quencher (Q) at the other. When LOCS probes are cleaved an increase in fluorescence above background can only be observed at high temperatures which are above the T_m of the stem region of a cleaved, split LOCS probe but below the T_m of the stem region of a uncleaved intact LOCS probe. Panel (iii) illustrates an M-Tec-P probe as described in Figures 1-4. These probes only result in an increase in fluorescence above background at low temperatures below the T_m OC1/OC2.

Figure 6 schematically illustrates an approach for multiplex analysis of two targets using the combination of one M-Tec-P Probe (A) and one LOCS probe (B) both of which are labelled with the same fluorophore (F) and quencher (Q). Reaction mixes contain an intact M-Tec-P Probe (Ai) and an intact LOCS probe (Bi). In the presence of target 1 (T1), PlexZyme 1 (P1) assembles and cleaves Ai to generate a cleaved M-Tec-P Probe (Ac). In the presence of target 2 (T2), PlexZyme 2 (P2) assembles and cleaves Bi to generate a cleaved, split LOCS Probe (Bc). Panel (i) illustrates structures which can form at temperature 1 which is below the T_m OC1/OC2 of both Ai and Ac, and below the T_m of the stem of both the Bi and Bc. Panel (ii) illustrates structures which can form at temperature 2 which is above the T_m OC1/OC2 of both Ai and Ac, and above the T_m of the stem of Bc but below the T_m of the stem of Bi. At temperature 1, fluorescence above background is generated in the presence of T1 but not in the absence of T1. At this temperature, Bi may get cleaved but no fluorescence above background is generated from Bc since the stem remains hybridized and the fluorophore remains quenched. Hence an increase in fluorescence above background at temperature 1 indicates the presence of T1 and background fluorescence is the same regardless of the presence or absence of T2. At temperature 2, the OC1 and OC2 of both Ai and Ac complexes dissociate and contribute to constant background fluorescence only. At this temperature, the stem of Bc, but not Bi,

dissociates resulting in an increase in fluorescence above background. Hence an increase in fluorescence above background at temperature 2 indicates the presence of T2 and background fluorescence is the same regardless of the presence or absence of T1.

Figure 7 Exemplary structures formed by components of an M-Tec-H and fluorescence at various temperatures in the presence and absence of target. In all panels, unhydrolyzed or hydrolyzed first oligonucleotide components OC1s are black and labelled with a quencher (Q); second oligonucleotide components OC2s are grey and labelled with a fluorophore (F) which is either in a quenched state (circles) or an unquenched/fluorescent state (stars); PCR primers are grey arrows; target nucleic acids are white with a grey outline. Low temperatures are below the T_m for hybridization of the capture region of OC1 to OC2 (T_m OC1/OC2) whilst high temperatures are above the T_m OC1/OC2. Panel (i) illustrates reaction components at low temperatures in the presence of target. In the presence of target, the 5'-3' exonuclease activity of polymerase hydrolyses the OC1 during PCR resulting in generation of a first fragment and a second fragment and separation of the F and Q, which in turn results in an increase in fluorescence indicative of the presence of the target. At this temperature OC2 remains hybridized to the first fragment of the cleaved OC1. Panel (ii) illustrates reaction components at low temperatures in the absence of target. No hydrolysis of OC1 occurs and the M-Tec-H remains quenched due to close proximity of the F and Q. Panel (iii) illustrates reaction components at high temperatures in the presence of target. When a target is present, hydrolysis will have occurred at the lower temperature, and both hydrolyzed (and residual unhydrolyzed) OC1 will be present in the reaction but neither will be hybridized to OC2. The contribution to background fluorescent signal is present and constant due to separation of the F and Q. Panel (iv) illustrates reaction components at high temperatures in the absence of target. No hydrolysis of the OC1 occurs and OC1 and OC2 will not be hybridized. The contribution to background fluorescent signal is present and constant due to separation of the F and Q. During a reaction, at low temperatures, there will be an increase in fluorescence in the presence of target (i) but no increase in fluorescence in the absence of target (ii). At high temperatures, the level of fluorescence is constant regardless of the presence (iii) or absence (iv) of target. The measured fluorescence arising from M-Tec-H at high temperature is constant and provides a background reading which does not change during the reaction.

Figure 8 schematically illustrates an approach for multiplex analysis of two targets using the combination of one M-Tec-H Probe (A) and one LOCS probe (B) both of which are labelled with the same fluorophore (F) and quencher (Q). Reaction mixes contain an

intact M-Tec-H Probe (Ai) and an intact LOCS probe (Bi). In the presence of target 1 (T1), the 5'-3' exonuclease activity of polymerase hydrolyses Ai to generate a cleaved M-Tec-H Probe (Ac). In the presence of target 2 (T2), a PlexZyme (P) assembles and cleaves Bi to generate a cleaved LOCS Probe (Bc). Panel (i) illustrates structures which can form at temperature 1 which is below the T_m OC1/OC2 of both Ai and Ac, and below the T_m of the stem of both the Bc and Bi. Panel (ii) illustrates structures which can form at temperature 2 which is above the T_m OC1/OC2 of Ai and Ac, and above the T_m of the stem of the Bc but below the T_m of the stem of the Bi. At temperature 1, fluorescence above background is generated by Ac in the presence of T1 but not by Ai in the absence of T1. At this temperature, Bi may get cleaved but no fluorescence above background is generated from Bc since the stem remains hybridized and the fluorophore remains quenched. Hence an increase in fluorescence above background at temperature 1 indicates the presence of T1 and background fluorescence is the same regardless of the presence or absence of T2. At temperature 2, OC1 and OC2 of both Ai and Ac complexes dissociate and contribute to a constant level of background fluorescence only. At this temperature the stem of Bc, but not Bi, dissociates resulting in an increase in fluorescence above background. Hence an increase in fluorescence above background at temperature 2 indicates the presence of T2 and background fluorescence is the same regardless of the presence or absence of T1.

Figure 9 An exemplary stem-loop LOCS reporter and its melting temperatures (T_m) in the Intact and Split conformations are illustrated. A LOCS reporter as exemplified can be used in combination with M-Tec Probes and other various standard reporter probes and substrates well known in the art for detection of nucleic acids. Exemplary intact LOCS reporters (A, LHS; top and bottom) have a Loop region which can be cleaved or degraded, a Stem region and detection moieties, for example a fluorophore (F) quencher (Q) dye pair. Cleavage or degradation of the Loop region in the presence of target can produce Split LOCS reporter structures (B, RHS; top and bottom). The T_m of the stem regions of the Intact LOCS (T_m A) is higher than the T_m of the stem regions in Split LOCS (T_m B). As such, the Stem of the Intact LOCS will melt and separate at temperatures at or above T_m A. In contrast, the stem holding the two fragments of the Split LOCS will melt and separate at temperatures at or above T_m B resulting in increased fluorescence.

Figure 10 illustrates an exemplary strategy for detection of a target using LOCS oligonucleotides which are universal and can be used to detect any target. In this scheme the LOCS oligonucleotide contains a stem region, a fluorophore quencher dye pair and a

loop region. The loop region comprises a universal substrate for a catalytic nucleic acid for example a PlexZyme, also known in the art and referred to herein as a MNAzyme. PlexZymes form when target sensor arms of component partzymes align adjacently on a target. The loop region of the LOCS oligonucleotide binds to the substrate binding arms of the assembled PlexZyme and the substrate within the LOCS loop is cleaved by the PlexZyme to generate a cleaved Split LOCS structure. Both the Intact LOCS and the Split LOCS will be either quenched, or will generate fluorescence, depending upon whether the temperature of the reaction milieu is above or below the melting temperature of their stems, namely T_m A and T_m B respectively. The presence of increases in fluorescence at temperatures greater than T_m B, but less than T_m A, is indicative of the presence of the target which facilitates the cleavage. The target can be directly detected, or target amplicons produced by target amplification protocols, can be detected.

Figure 11 shows amplification curves where fluorescence was acquired at 52°C (LHS) and 76°C (RHS) for reactions containing an M-Tec-P specific to *Chlamydia trachomatis* (CT) in the presence of various numbers of gene copies (10000 (A), 400 (B) or 10 (C)) or no target (D).

Figure 12 shows reactions where *Trichomonas vaginalis* (TV) target is detected using either M-Tec-P probes (MT) or Molecular Beacons (MB). Amplification plots are shown for reactions mediated by AptaTaq exo DNA polymerase (A) or AptaTaq DNA polymerase (B) with acquisition at 52°C (MT-52 or MB-52) or at 76°C (MT-76 or MB-76). Reactions contained either 10,000 copies of target (i), 100 copies of target (ii) or no target (iii).

Figure 13 shows reactions which use the same pair of universal probes, one M-Tec-P probe and one LOCS Probe, in two separate reactions to simultaneously detect two targets at two temperatures at the same wavelength during PCR.

Reaction Mix 1 included reagents (primers, partzymes etc) plus an M-Tec-P Probe which detected *Trichomonas vaginalis* (TV) at 52°C (A) and a LOCS probe which detected the TFRC in human genomic DNA (gDNA) at 76°C (B). Reaction Mix 2 included reagents (primers, partzymes etc) plus the same M-Tec-P Probe used in Reaction Mix 1 which detected *Neisseria gonorrhoeae* (NG) at 52°C (C) and the same LOCS probe used in Reaction Mix 1 which detected *Chlamydia trachomatis* (CT) at 76°C (D).

The figure shows real time amplification curves generated using an M-Tec-P probe and a LOCS probe with acquisition in the FAM channel at 52°C (A and C) and 76°C (B and D) in the presence of Mix 1 and 10000, 800 or 40 copies of TV only (black solid

line), or both TV and TFRC in gDNA (grey dashed line), or TFRC in gDNA only (black dashed line), or no template (grey solid line) A and B); or in the presence of mix 2 and 10000, 800 or 40 copies of NG only (black solid line), or CT only (black dashed line), or both NG and CT (grey dashed line) or no target (grey solid line) (C and D).

Figure 14 shows real time amplification curves generated using an M-Tec-H probe with acquisition in the HEX channel at 52°C (A) and 76°C (B) in the presence of 10000 (i) or 40 copies (ii) of the target MG, or in the absence of target (iii).

Figure 15 shows real time amplification curves generated in a single reaction using a Molecular Beacon (MB) for detection of TV in FAM channel (A), LOCS probe 1 for detection of NGopa in FAM channel (B), M-Tec-P probe (MT) for detection of CT in VIC channel (C), LOCS probe 2 for detection of MG in VIC channel (D) and Linear PlexZyme substrate (LS) for detection of NGporA in CY5 channel (E), with acquisition at 52°C (top row) or at 76°C (bottom row). Reactions contained either 10000 copies of target (a), 1000 copies of target (b) 100 copies of target (c) or no target (d).

Figure 16 illustrates exemplary components for an M-Tec-E probe suitable for hydrolysis by a restriction endonuclease, for example a Nicking enzyme, in the presence of a target. This M-Tec-E has two oligonucleotide components namely a first oligonucleotide component OC1 and a second oligonucleotide component OC2. OC1 is labelled with a quencher at one terminus and contains a sensor region that is both complementary to the target and inclusive of one strand of a double stranded recognition site for the restriction endonuclease. OC2 includes a fluorophore at one terminus and a region which is capable of hybridizing to the capture region of OC1.

Figure 17 illustrates exemplary components and structures for M-Tec-P Probe complexes composed of (i) three oligonucleotide components, OC1, OC2 and OC3, (ii) Partzymes A and B, and (iii) target template; where (iv) shows the complex formed when an M-Tec-P Probe binds to a PlexZyme assembled in the presence of the target. In this complex, OC1 contains a substrate amenable to cleavage by a PlexZyme. OC1 is connected to a detection moiety but is not directly labelled with the detection moiety. The components OC2 and OC3 contain non-target sequences complementary to respective first and second capture regions of OC1 and are labelled with fluorophore and quencher dye pairs. The melting temperature of the complementary regions of OC1 and OC2 (T_m OC1/OC2) could be lower than the melting temperature of the complementary regions of OC1 and OC3 (T_m OC1/OC3).

At a first temperature below T_m OC1/OC2, both OC2 and OC3 would bind to the intact OC1 resulting in quenched complexes. If OC1 were cleaved by a PlexZyme assembled in the presence of target, OC2 and OC3 would remain bound to the first and second fragments of OC1 respectively; however, the two detection moieties would separate and an increase in fluorescence above the baseline would be measurable at this temperature. At a second temperature above T_m OC1/OC2, OC2 would dissociate from both intact OC1 and/or cleaved OC1 first fragments, separating the two detection moieties so that a constant level of fluorescence would contribute to background regardless of whether or not the target had been present in the reaction.

Figure 18 Exemplary components and structures for M-Tec-H Probe complexes comprising three oligonucleotide components, OC1, OC2 and OC3 together with a 5' primer, a polymerase with inherent 5'-3' exonuclease activity (A). In this complex, OC1 contains a sensor region, at least a portion of which is complementary to the target. OC1 is connected to a detection moiety but is not directly labelled with the detection moiety. The components OC2 and OC3 contain non-target sequences complementary to respective first and second capture regions of OC1 and could be labelled with fluorophore and quencher dye pairs. The melting temperature of the complementary regions of OC1 and OC2 (T_m OC1/OC2) could be lower than the melting temperature of the complementary regions of OC1 and OC3 (T_m OC1/OC3).

At a first temperature below T_m OC1/OC2, both OC2 and OC3 would bind to the intact OC1 resulting in quenched complexes. If OC1 were hydrolysed by exonuclease activity in the presence of target, OC2 and OC3 would remain bound to the first and second fragments of OC1; however, the two detection moieties would separate and an increase in fluorescence above the baseline would be measurable at this temperature. At a second temperature above T_m OC1/OC2, OC2 would dissociate from both intact OC1 and/or hydrolysed OC1 first fragments, separating the two detection moieties so that a constant level of fluorescence would be contributed to background regardless of whether or not the target had been present in the reaction.

Exemplary components and structures for M-Tec-E Probe complexes comprising three oligonucleotide components, OC1, OC2 and OC3 together with a nicking endonuclease (B). In this complex, OC1 contains a sensor region, at least a portion of which is complementary to the target. OC1 is connected to a detection moiety but is not directly labelled with the detection moiety. The components OC2 and OC3 would contain non-target sequences complementary to respective first and second capture regions of

OC1 and would be labelled with fluorophore and quencher dye pairs. The melting temperature of the complementary regions of OC1 and OC2 (T_m OC1/OC2) could be lower than the melting temperature of the complementary regions of OC1 and OC3 (T_m OC1/OC3). Hybridization of the target with the sensor region of OC1 would create the double stranded recognition sequence for the nicking endonuclease.

At a first temperature below T_m OC1/OC2, both OC2 and OC3 would bind to the intact OC1 resulting in quenched complexes. If OC1 were nicked/cleaved by the endonuclease activity in the presence of target, OC2 and OC3 would remain bound to the first and second fragments of OC1; however, the two detection moieties would separate and an increase in fluorescence above the baseline would be measurable at this temperature. At a second temperature above T_m OC1/OC2, OC2 would dissociate from both intact OC1 and/or nicked OC1 first fragments, separating the two detection moieties so that a constant level of fluorescence would be contributed to background regardless of whether or not the target had been present in the reaction.

Figure 19 Illustration of two M-Tec-P probes that could be used for a single channel multiplexing. Both M-Tec-P probe A and M-Tec-P probe B could be composed of two oligonucleotide components (OC1 and OC2) with both probes labelled with the same fluorophore (F) and quencher (Q) dye pair. Each of the OC1 within M-Tec-P probe A and M-Tec-P probe B could contain different substrates specific for two PlexZymes capable of assembling in the presence of a first target (T1) or a second target (T2) respectively. The melting temperature of the two oligonucleotide components (T_m OC1/OC2) of the M-Tec-P probe A would be lower than that of M-Tec-P probe B.

Figure 20 Illustrated exemplary melt curve analysis charts that could be generated for reactions containing two M-Tec Probes where an M-Tec probe A has a lower T_m OC1/OC2 than a second M-Tec probe B. Illustrative diagrams show the change in the first derivative of RFU over a temperature range that would be generated following incubation in the absence of both a first and a second target (Figure 20A), in the presence of both a first and a second target (Figure 20B), in the presence of a first target and absence of a second target (Figure 20C), and in the presence of a second target and absence of a first target (Figure 20D). The local minima in the chart would represent the T_m OC1/OC2 of each M-Tec probe, which could be observed in the absence of the specific target in the reaction, but no longer could be observed in the presence of the target(s).

Figure 21 shows an endpoint qualitative analysis by displaying the difference in fluorescence before and after PCR (Δ RFU) in reactions containing an M-Tec-P probe and a LOCS probe at 52°C (**A**) and 76°C (**B**); and reactions containing a Molecular Beacon and a LOCS probe at 52°C (**C**) and 76°C (**D**). The graphs are displayed as mean of triplicates of reactions containing either 10000, 800 or 40 copies of CT only, NG only, or both NG and CT or no target (NTC). The error bars denote the standard deviation.

Figure 22 illustrates an example of an M-Tec-P probe comprising OC1 and OC2 oligonucleotides, and an Aptazyme. The OC1 oligonucleotides could be designed to contain a quencher, a capture region complementary to a sequence of the OC2 oligonucleotide and a sensor region that may function as a substrate for a specific DNAzyme. The complementary region between the OC1 and OC2 oligonucleotides could be designed to have a melting temperature being higher than a first temperature, but lower than a second temperature, where fluorescence measurements would be made. The OC2 oligonucleotide could be designed to contain a fluorophore. The Aptazyme could be designed to contain an aptamer region with specific affinity to a target, a DNAzyme region that is capable of cleaving the substrate sequence in the sensor region of OC1, and a cDNA region containing complementary sequences to the aptamer region. In the absence of target the DNAzyme could be held in an inactive conformation due to binding of the cDNA to the aptamer region. Binding of the specific target to the aptamer could induce dissociation of the cDNA/aptamer double stranded region, which allows activation of the DNAzyme. This DNAzyme could then cleave the M-Tec-P probe and generate a detectable signal that is measurable at temperatures below the T_m OC1/OC2.

Figure 23 illustrates analysis of fluorescence data acquired in the VIC channel where M-Tec-P was used for direct detection of a nucleic acid target (TV), in the absence of target amplification. Endpoint detection of the target, which was calculated as the difference between the fluorescence signal acquired before and after the isothermal incubation with partzymes specific for the target, showed that there was a significant increase in fluorescence signal only in the presence of the target, but not in the absence at 52°C (**A**). In contrast, there was no significant increase in fluorescence regardless of the presence or absence of the target at 76°C (**B**). The real-time fluorescence data acquired during the isothermal step at 52°C (**C**), indicate the target-dependent increase in fluorescence at concentrations of 125 pM (solid grey line), 25 pM (dashed grey line) and 12.5 pM (dotted grey line), but no significant increase in the absence of the target (solid black line). The derivative melt curve analysis from 40°C to 95°C acquired after the

isothermal incubation (D) shows there is a melt signature curve with a peak at 61.5°C in reactions with no target template (black solid line), but this is not observed in any of the reactions containing the target (solid, dashed or dotted grey line).

Definitions

As used in this application, the singular form “a”, “an” and “the” include plural references unless the context clearly dictates otherwise. For example, the phrase “polynucleotide” also includes a plurality of polynucleotides.

As used herein, the term “comprising” means “including”. Variations of the word “comprising”, such as “comprise” and “comprises,” have correspondingly varied meanings. Thus, for example, a polynucleotide “comprising” a sequence of nucleotides may consist exclusively of that sequence of nucleotides or may include one or more additional nucleotides.

As used herein the term “plurality” means more than one. In certain specific aspects or embodiments, a plurality may mean 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, or more, and any integer derivable therein, and any range derivable therein.

As used herein, the term “subject” includes any animal of economic, social or research importance including bovine, equine, ovine, primate, avian and rodent species. Hence, a “subject” may be a mammal such as, for example, a human or a non-human mammal. Also encompassed are microorganism subjects including, but not limited to, bacteria, archaea, viruses, fungi/yeasts, protists and nematodes. A “subject” in accordance with the present invention also includes infectious agents such as prions. A subject may also include an algae or a plant.

As used herein, the terms “polynucleotide” and “nucleic acid” may be used interchangeably and refer to a 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, LNA, PNA 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 comprising synthetic, mammalian, human, animal, plant, fungal, bacterial, viral, archaeal or any combination thereof.

As used herein, the term “oligonucleotide” refers to a segment of DNA or a DNA-containing nucleic acid molecule, or RNA or RNA-containing molecule, or a combination thereof. Examples of oligonucleotides include nucleic acid targets; component for M-Tec, substrates, for example, those which can be modified by an PlexZyme; primers such as those used for *in vitro* target amplification by methods such as PCR; components of PlexZymes; and various other types of reporter probes or systems, including but not limited to, TaqMan or Hydrolysis probes; Molecular Beacons; Sloppy Beacons; Eclipse probes; Scorpion Uni-Probe, Scorpion Bi-Probes primer/probes, Double-stranded probes (Yin-Yang probes), Catchers and Pitchers used in TOCE technology and dual-hybridization probes. The term “oligonucleotide” includes reference to any specified sequence as well as to the sequence complementary thereto, unless otherwise indicated. Oligonucleotides may comprise at least one addition or substitution, including but not limited to the group comprising 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 2'-O-methylcytidine, 5-carboxymethylaminomethyl thioridine, 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, beta D-arabinosyl thymidine.

The terms “polynucleotide”, “nucleic acid” and “oligonucleotide” include reference to any specified sequence as well as to the sequence complementary thereto, unless otherwise indicated.

As used herein, the terms “complementary”, “complementarity”, “match” and “matched” refer to the capacity of nucleotides (e.g. deoxyribonucleotides, ribonucleotides or combinations thereof) to hybridise to each other via Watson-Crick base-pairing, noncanonical base-pairing including wobble base-pairing and Hoogsteen base-pairing

(e.g. LNA, PNA or BNA) or unnatural base pairing (UBP). Bonds can be formed via Watson-Crick base-pairing between adenine (A) bases and uracil (U) bases, between adenine (A) bases and thymine (T) bases, between cytosine (C) bases and guanine (G) bases. A wobble base pair is a noncanonical base pairing between two nucleotides in a polynucleotide duplex (e.g. guanine-uracil, inosine-uracil, inosine-adenine, and inosine-cytosine). Hoogsteen base pairs are pairings that, like Watson-Crick base pairs, occur between adenine (A) and thymine (T) bases, and cytosine (C) and guanine (G) bases, but with differing conformation of the purine in relation to the pyrimidine compared to in Watson-Crick base pairings. An unnatural base pair is a manufactured subunit synthesized in the laboratory and not occurring in nature. Nucleotides referred to as “complementary” or that are the “complement” of each other are nucleotides which have the capacity to hybridise together by either Watson-Crick base pairing or by noncanonical base pairing (wobble base pairing, Hoogsteen base pairing) or by unnatural base pairing (UBP) between their respective bases. A sequence of nucleotides that is “complementary” to another sequence of nucleotides herein may mean that a first sequence is 100% identical to the complement of a second sequence over a region of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more nucleotides. Reference to a sequence of nucleotides that is “substantially complementary” to another sequence of nucleotides herein may mean that a first sequence is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98% or 99% identical to the complement of a second sequence over a region of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more nucleotides.

As used herein, the terms “non-complementary”, “not complementary”, “mismatch” and “mismatched” refer to nucleotides (e.g. deoxyribonucleotides, ribonucleotides, and combinations thereof) that lack the capacity to hybridize together by either Watson-Crick base pairing or by wobble base pairing between their respective bases. A sequence of nucleotides that is “non-complementary” to another sequence of nucleotides herein may mean that a first sequence is 0% identical to the complement of a second sequence over a region of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more nucleotides.

Reference to a sequence of nucleotides that is “substantially non-complementary” to another sequence of nucleotides herein may mean that a first sequence is less than 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, or 40% identical to the complement of a second sequence over a region of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19,

20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more nucleotides.

As used herein, the term “target” refers to any molecule or analyte present in a sample that the methods of the present invention may be used to detect. The term “target” will be understood to include nucleic acid targets, and non-nucleic acid targets such as, for example proteins, peptides, analytes, ligands, and ions (e.g. metal ions).

As used herein, an “enzyme” refers to any molecule which can catalyze a chemical reaction (e.g. amplification of a polynucleotide, cleavage of a polynucleotide etc.). Non-limiting examples of enzymes suitable for use in the present invention include nucleic acid enzymes and protein enzymes. Non-limiting examples of suitable nucleic acid enzymes include ribozymes, MNazymes (i.e., PlexZymes), deoxyribozymes (i.e. DNAzymes) and aptazymes. Non-limiting examples of suitable protein enzymes include polymerases, reverse transcriptase, exonucleases and endonucleases. The enzymes will generally provide catalytic activity that assists in carrying out one or more of the methods described herein. By way of non-limiting example, the exonuclease activity may be an inherent catalytic activity of, for example, a polymerase. By way of non-limiting example, the endonuclease activity may be an inherent catalytic activity of, for example, a restriction enzyme including a Nicking endonuclease, a riboendonuclease or a duplex specific nuclease (DSN).

As used herein, an “amplicon” refers to nucleic acid (e.g. DNA or RNA, or a combination thereof) that is a product of natural or artificial nucleic acid amplification or replication events including, but not limited to PCR, RT-PCR, SDA, NEAR, HDA, RPA, LAMP, RCA, TMA, LCR, RAM, 3SR, NASBA, and any combination thereof.

As used herein, the term “stem-loop oligonucleotide” will be understood to mean a DNA or DNA-containing molecule, or an RNA or RNA-containing molecule, or a combination thereof (i.e. DNA-RNA hybrid molecule or complex), comprising or consisting of a double-stranded stem component joined to a single-stranded loop component. The double-stranded stem component comprises a forward strand hybridized by complementary base pairing to a complementary reverse strand, with the 3' nucleotide of the forward strand joined to the 5' nucleotide of the single-stranded loop component, and the 5' nucleotide of the reverse strand joined to the 3' nucleotide of the single-stranded loop component. The two strands of the stem need not necessarily form a blunt ended structure. There may be additional bases which result in a single stranded overhang and these overhanging bases still provide suitable sites for attachment of detection moieties. The double-stranded stem component may comprise one or more detection

moieties, including but not limited to, a fluorophore on one strand (e.g. the forward strand), and one or more quenchers on the opposing strand (e.g. the reverse strand). Other non-limiting examples include a gold or silver nanoparticle on both strands for colorimetric detection, immobilization of one strand to a gold surface (e.g. the forward strand) and a gold nanoparticle on the opposing strand (e.g. the reverse strand) for SPR detection, and immobilization of one strand to an electrode surface (e.g. the forward strand) and a methylene blue molecule on the opposing strand (e.g. reverse strand) for electrochemical detection.

As used herein, the terms “M-Tec Probe” and/or “M-Tec Substrate” and/or “M-Tec complex” and/or “Multi-component Temperature-Controlled probe”, are used interchangeably to mean a Multiple-component Temperature-Controlled probe, substrate or complex. As used herein M-Tec Probes may comprise two or more oligonucleotide components. M-Tec Probes comprising at least two oligonucleotide components may have a “first oligonucleotide component” and a “second oligonucleotide component”. The terms “first oligonucleotide component” or “first component oligonucleotide” or “OC1” may be used interchangeably to mean a first component molecule of an M-Tec probe. The terms “second oligonucleotide component” or “second component oligonucleotide” or “OC2” may be used interchangeably to mean a second component molecule of an M-Tec probe. The terms “third oligonucleotide component” or “third component oligonucleotide” or “OC3” may be used interchangeably to mean a third component molecule of an M-Tec probe.

The first oligonucleotide component comprises a “sensor region” that can serve as a substrate for an enzyme only in the presence of the target to be detected. The first oligonucleotide component also comprises a first “capture region” which is complementary to the second oligonucleotide component. In some embodiments, the first oligonucleotide component is connected to a single detection moiety which may be, for example, either a fluorophore or a quencher. Optionally, the first oligonucleotide component may further comprise a second “capture region” which is complementary to the third oligonucleotide component. Preferably, the capture region does not overlap with the sensor region. The regions of complementarity between the first and second oligonucleotide components may be capable of hybridization or association at temperatures below the melting temperatures of the complementary regions of the first and second oligonucleotide components. The first and third oligonucleotide components may have regions of complementarity which may be capable of hybridization or

association at temperatures below the melting temperatures of the complementary regions of the first and third oligonucleotide components.

The term “T_m OC1/OC2” as used herein refers to the melting temperature of the complementary regions of the first and second oligonucleotide components. The term “T_m OC1/OC3” as used herein refers to the melting temperature of the complementary regions of the first and third oligonucleotide components. The first oligonucleotide component may be connected to a first detection moiety, and the second oligonucleotide component may be connected to a second detection moiety. In some examples, the first oligonucleotide component is directly labelled with (i.e., directly attached to, such as covalently attached to) a first detection moiety and the second oligonucleotide component is directly labelled with (i.e., directly attached to, such as covalently attached to) a second detection moiety. In other examples, the first oligonucleotide component is connected to the first detection moiety via a third oligonucleotide component, wherein the first oligonucleotide component further comprises a second capture region capable of hybridization to the third oligonucleotide component by complementary base pairing, and wherein the third oligonucleotide component is directly labelled with (i.e., directly attached to, such as covalently attached to) the first detection moiety. In such examples, it will be understood that the first oligonucleotide component is nevertheless connected to the first detection moiety (via hybridization to the third oligonucleotide component) but it is not directly labelled with the first detection moiety. Accordingly, the first oligonucleotide component may be connected to the first detection moiety either directly (i.e., directly labelled) or indirectly (e.g., via a third oligonucleotide component, which itself is directly labelled with the first detection moiety).

If present, the third oligonucleotide component may be capable of hybridization with the first oligonucleotide components at temperatures below the T_m OC1/OC3 which is greater than the T_m OC1/OC2. As such, at temperatures below T_m OC1/OC2 all oligonucleotide components in a complex will be hybridized. The first and second detection moieties may be, for example, a fluorophore and a quencher or vice versa.

The first oligonucleotide component may be capable of modification in the presence of a target molecule to be detected. Modification may be mediated by enzymatic activity. The modification by enzymatic activity may be, for example, cleavage by a PlexZyme and/or hydrolysis by the exonuclease activity of a polymerase and/or cleavage by a restriction endonuclease, for example a nicking endonuclease. Some M-Tec Probes incorporate first oligonucleotide components which comprise nucleic acid enzyme

substrates which may be universal, and which are capable of catalytic cleavage by nucleic acid enzymes such as PlexZymes, DNAzymes, ribozymes and aptazymes.

Enzymatic modification of an oligonucleotide substrate may include cleavage or hydrolysis by a protein enzyme or a catalytic nucleic acid. Cleavage and/or hydrolysis may be referred to as digestion. Similarly, cleaving and/or hydrolysing in this context may be referred to as digesting. In some examples, the first oligonucleotide component is not connected to a separate blocking group, wherein the blocking group is not a fluorophore or a quencher. In some examples, the second oligonucleotide component is not connected to a separate blocking group, wherein the blocking group is not a fluorophore or a quencher.

As used herein the terms “Intact M-Tec” probe and “unmodified M-Tec” probe are used interchangeably to refer to an M-Tec probe wherein the first oligonucleotide component of the complex has not been modified by cleavage, hydrolysis or nicking by an enzyme. The terms “Cleaved M-Tec” probe or “Hydrolyzed M-Tec” probe or “modified M-Tec” probe are used to refer to an M-Tec probe wherein the first oligonucleotide component of the probe complex has been modified by cleavage, nicking or hydrolysis by an enzyme in the presence of target. The terms “Intact first oligonucleotide component”, “Intact OC1”, “Intact first component oligonucleotide”, “unmodified first oligonucleotide component”, “unmodified OC1” and “unmodified first component oligonucleotide” are used interchangeably to refer to a first oligonucleotide component which has not been modified by cleavage, hydrolysis or nicking by an enzyme. The terms “Cleaved first oligonucleotide component”, “Cleaved OC1”, “Cleaved first component oligonucleotide”, “Hydrolyzed first oligonucleotide component”, “Hydrolyzed OC1”, “Hydrolyzed first component oligonucleotide”, “modified first oligonucleotide component”, “modified OC1” and “modified first component oligonucleotide” are used to refer to a first oligonucleotide component which has been modified by cleavage, hydrolysis or nicking by an enzyme. Modification of a first oligonucleotide component can generate multiple “first oligonucleotide component fragments”, “OC1 fragments”, “first component oligonucleotide fragments”, “fragments of the first oligonucleotide components”, “fragments of the first component oligonucleotides” or “fragments of the OC1”. Cleavage or hydrolysis of substrates which are present within the first oligonucleotide component of an Intact M-Tec probe complex by an enzyme may generate multiple fragments of the first oligonucleotide component which are associated with modified M-Tec probes.

In some embodiments, the position or region of the first oligonucleotide component which is modified by cleavage, hydrolysis or nicking by an enzyme is located between the first capture region and the connected first detection moiety.

Various types of M-Tec Probes are disclosed herein. The term “M-Tec-P” probe as used herein refers to an M-Tec complex that may be suitable for cleavage by a catalytic nucleic acid such as a PlexZyme assembled in the presence of a target. An M-Tec-P probe comprising at least two oligonucleotides may have a first oligonucleotide component comprising, within its sensor region, a PlexZyme substrate region. An M-Tec-P probe may be suitable for cleavage by other types of catalytic nucleic acids such as DNAzymes, aptazymes or ribozymes which are only activated in the presence of target. An M-Tec-P probe comprising at least two oligonucleotides may have a first oligonucleotide component comprising, within its sensor region, a substrate region for a catalytic nucleic acid such as a DNAzyme, an aptazyme or a ribozyme. The term “M-Tec-H” probe as used herein refers to an M-Tec complex that may be suitable for hydrolysis by exonuclease activity, for example, by intrinsic 5' to 3' exonuclease activity of polymerase in the presence of target. An M-Tec-H probe comprising at least two oligonucleotides may have a first oligonucleotide component which comprises, within its sensor region, a sequence which is complementary to the target. The first oligonucleotide component may be capable of binding or hybridising to the target at a location which is 3' of the upstream forward PCR primer. The term “M-Tec-E” probe as used herein refers to an M-Tec complex that may be suitable for cleaving or nicking by an endonuclease, for example, by a nicking endonuclease. An M-Tec-E probe comprising at least two oligonucleotides may have a first oligonucleotide component which comprises, within its sensor region, a sequence which is complementary to the target. The first oligonucleotide component may be capable of hybridizing to the target and forming a double stranded recognition sequence for a specific endonuclease.

As used herein, the term “capture region” refers to a region of the first oligonucleotide component which hybridises to the second oligonucleotide component or to the region of the first oligonucleotide component which hybridises to a third oligonucleotide component if one is present.

As used herein, the term “universal M-Tec-P probe” refers to a M-Tec structure which contains a first “universal component oligonucleotide” with a first “universal capture region”, and a “universal sensor region” which comprises a universal catalytic nucleic acid substrate which can be cleaved by any PlexZyme with complementary substrate binding arms regardless of the sequences of the PlexZyme target sensing arms.

The catalytic nucleic acid substrate is not complementary to the target and hence is universal since it may be linked to any target via the incorporation of target specific partzymes. A single universal M-Tec-P probe can be used as a surrogate marker for any target which is capable of facilitating the cleavage of a specific M-Tec-P probe. A series of universal M-Tec-P probes can be incorporated into any multiplex assay designed to analyze any set of targets.

Additionally, the first universal oligonucleotide component may be connected to a first detection moiety which may be a quencher molecule that has the capability of quenching a range of different fluorescent detection moieties. In such cases, the first universal capture region of the first oligonucleotide component may be capable of hybridization to any of a series of second oligonucleotide components, each of which has the same sequence but is labelled with a different fluorescent detection moiety that may be quenched by the quencher connected to the first oligonucleotide component.

Alternatively, a series of first oligonucleotide components for multiplexing or detection of multiple targets may be connected to different first detection moieties, each of which may be a different fluorophore molecule, and each in the series may further comprise a universal capture region. In such cases, the universal capture region of the first oligonucleotide component may be capable of hybridization to a single universal second oligonucleotide component, which is labelled with a quencher detection moiety that may quench the multiple fluorophores connected to different first oligonucleotide components.

Alternatively, or additionally, if the first oligonucleotide component has first and a second capture regions, both of which are universal and capable of hybridization with universal second and third oligonucleotide components respectively, then the first oligonucleotide component can be universal with respect to both detection moieties (e.g. the fluorophore and the quencher), as well as with the orientation of these. The sequences of the second and third oligonucleotide components could remain the same; however, manufacturers and assay developers would have freedom of choice with respect to which fluorophore and quencher molecules can be connected to the second and third oligonucleotide components. These could be used in conjunction with a first oligonucleotide component which is not directly labelled with any detection moieties.

Finally, M-Tec-P probes comprising nucleic acid enzyme substrates within the sensor regions may be universal with respect to the type of catalytic nucleic acid which can cleave them. By way of example, the same nucleic acid enzyme substrate sequence may be cleaved by PlexZymes, aptazymes, and/or DNAzymes. By way of further example, a nucleic acid enzyme substrate sequence, suitable for cleavage with 10:23 DNAzyme,

could also be cleaved by an aptazyme incorporating a 10:23 DNAzyme or by a PlexZyme composed of partzymes harboring partial catalytic core sequences homologous to regions of the 10:23 DNAzyme.

As used herein, the term “stem-loop oligonucleotide” will be understood to include “LOCS”, also referred to herein as a “LOCS oligonucleotide”, “LOCS structure”, “LOCS reporter”, “Intact LOCS”, “LOCS probes” and “PlexPlus Probes”. The single-stranded loop component of a LOCS may comprise a region capable of serving as a substrate for a catalytic nucleic acid such as, for example, an MNAzyme (i.e., a PlexZyme), a DNAzyme, a ribozyme, an apta-PlexZyme, or an aptazyme. Additionally, or alternatively, the single-stranded loop component may comprise a region which is complementary to a target nucleic acid (e.g. a target for detection, quantification and the like), and/or amplicons derived therefrom, and which may further be capable of serving as a substrate for an exonuclease enzyme. By way of non-limiting example, the exonuclease may be an inherent activity of a polymerase enzyme. Additionally, or alternatively, the single-stranded loop component region may comprise a region which may: (i) be complementary to the target being detected, (ii) comprise one strand of a double stranded restriction enzyme recognition site; and (iii) be capable of serving as a substrate for a restriction enzyme, for example a nicking endonuclease. As used herein, the terms “split stem-loop oligonucleotide”, “split LOCS”, “split LOCS oligonucleotide”, “split LOCS structure”, “split LOCS reporters”, “split LOCS probes”, “cleaved LOCS” and “degraded LOCS” are used herein interchangeably and will be understood to be a reference to a “LOCS” in which the single-stranded loop component has been cleaved, digested, and/or degraded (e.g. by an enzyme as described herein) such that at least one bond between adjacent nucleotides within the loop is removed, thereby providing a non-contiguous section in the loop region. In split LOCS, the forward and reverse strands of the double-stranded stem portion may retain the ability to hybridize to each other to form a stem in a temperature-dependent manner.

LOCS are designed to include a cleavable loop region enabling target-dependent cleavage of the loop region by an enzyme generating a split LOCS. This in turn may facilitate detection of the target from a detectable signal generated at specific temperature(s) following association (hybridization) or dissociation of the stem portion of intact or split LOCS. In contrast, a Molecular Beacon as used herein refers to a stem loop oligonucleotide designed to include a loop region that is not cleavable during the methods described herein. Molecular Beacons may mediate target detection by generating detectable signal at specific temperatures following association (hybridization) or

dissociation (separation) of the loop portion of the probe with the target to be detected. As such, a primary difference between these two types of stem loop structures in the context of the present invention is that LOCS are monitored by measuring changes in signals due to hybridization or dissociation of the stem region of intact or split LOCS, whereas Molecular Beacons are monitored by measuring changes in signal due to hybridization or dissociation of the loop region and the target.

As used herein, the term “universal stem” refers to a double stranded sequence which can be incorporated into any LOCS structure. The same “universal stem” may be used in LOCS which contain Loops which comprise either catalytic nucleic acid substrates or sequence which is complementary to a target of interest. A single universal stem can be used as a surrogate marker for any target which is capable of facilitating the splitting of a specific LOCS. A series of universal stems can be incorporated into a series of LOCS designed for analysis of any set of targets.

As used herein, the term “universal LOCS” refers to a LOCS structure which contains a “universal stem”, and a “universal Loop” which comprises a universal catalytic nucleic acid substrate which can be cleaved by any PlexZyme with complementary substrate binding arms regardless of the sequences of the PlexZyme target sensing arms. A single universal LOCS can be used as a surrogate marker for any target which is capable of facilitating the splitting of a specific LOCS. A series of universal LOCS can be incorporated into any multiplex assay designed to analyse any set of targets.

Some LOCS probes comprise nucleic acid enzyme substrates within the loop regions which may be universal, and which are capable of catalytic cleavage by nucleic acid enzymes such as PlexZymes, DNAzymes and aptazymes. Other LOCS probes comprise target specific sequences within the loop region which are capable of catalytic cleavage by protein enzymes including endonucleases and exonucleases.

As used herein, 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-containing molecule or complex, or a combination thereof (i.e. DNA-RNA hybrid molecule or complex), which may recognize at least one substrate and catalyse a modification (such as 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 may comprise a single DNA or DNA-containing molecule (also known in the art as a “DNA enzyme”, “deoxyribozyme” or “DNAzyme”) or an RNA or RNA-containing

molecule (also known in the art as a “ribozyme”) or a combination thereof, being a DNA-RNA hybrid molecule which may recognize at least one substrate and catalyse a modification (such as cleavage) of the at least one substrate. The 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 cleavage) of the at least one substrate. The terms “nucleic acid enzyme”, “catalytic nucleic acid”, “nucleic acid with catalytic activity”, “catalytic nucleic acid complex” and “catalytic nucleic acid enzyme” include within their meaning PlexZymes.

As used herein, the terms “PlexZymes”, “MNAzymes” and “multi-component nucleic acid enzyme” have the same meaning and refer to multi-component nucleic acid enzymes having two or more oligonucleotide sequences (e.g. partzymes) which, only in the presence of an PlexZyme assembly facilitator (for example, a target), form an active nucleic acid enzyme that is capable of catalytically modifying a substrate. The terms “PlexZyme”, “MNAzyme” and “multi-component nucleic acid enzyme” 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.

PlexZymes and MNAzymes can catalyse a range of reactions including cleavage of a substrate, and other enzymatic modifications of a substrate or substrates. Component partzymes A and B each bind to an assembly facilitator (e.g. a target DNA or RNA sequence) through base pairing. The PlexZyme only forms when the sensor arms of partzymes A and B hybridize adjacent to each other on the target assembly facilitator. The substrate arms of the PlexZyme engage the substrate, the modification (e.g. cleavage) of which is catalyzed by the catalytic core of the PlexZyme, formed by the interaction of the partial catalytic domains of partzymes A and B. PlexZymes may cleave DNA/RNA chimeric reporter substrates.

It will be understood that the terms “PlexZyme”, “MNAzyme” and “multi-component nucleic acid enzyme” as used herein encompass all known MNAzymes and modified MNAzymes 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 MNAzymes and modified MNAzymes encompassed by the terms “MNAzyme” and “multi-component nucleic acid enzyme” include

MNAzymes with cleavage catalytic activity (as exemplified herein), disassembled or partially assembled MNAzymes comprising one or more assembly inhibitors, MNAzymes comprising one or more aptamers (“apta-MNAzymes”), MNAzymes comprising one or more truncated sensor arms and optionally one or more stabilizing oligonucleotides, MNAzymes comprising one or more activity inhibitors, multi-component nucleic acid inactive proenzymes (MNAi), each of which is described in detail in one or more of WO/2007/041774, WO/2008/040095, US 2007-0231810, US 2010-0136536, and/or US 2011-0143338.

As used herein, the terms “partzyme”, “component partzyme” and “partzyme component” refer to a DNA-containing or RNA-containing or DNA-RNA-containing oligonucleotide, two or more of which, only in the presence of an PlexZyme assembly facilitator as herein defined, can together form an “PlexZyme.” 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. The terms “sensor arm”, “target sensor arm” or “target sensing arm” or “target arm” may be used interchangeably to describe the domain of the partzymes which binds to the assembly facilitator, for example the target. 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 stabilizes, the PlexZyme structure by interacting with either an assembly facilitator or a substrate.

The terms “assembly facilitator”, “PlexZyme assembly facilitator”, “MNAzyme assembly facilitator”, and “target assembly facilitator” as used herein refer to entities that can facilitate the self-assembly of component partzymes to form a catalytically active PlexZyme by interaction with the sensor arms of the PlexZyme. As used herein, assembly facilitators may facilitate the assembly of PlexZymes which have cleavage or other enzymatic activities. In some examples, an assembly facilitator is required for the self-assembly of a PlexZyme. An assembly facilitator may be comprised of a single molecule, or it 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 comprise one or more nucleotide component/s which do not share sequence complementarity with sensor arm/s of the PlexZyme. 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: PCR, RT-PCR, SDA, NEAR, HDA, RPA, LAMP, RCA, TMA, RAM, LCR, 3SR, or NASBA.

PlexZymes are capable of cleaving substrates incorporated into various probe types, including but not limited to, (i) linear substrates, (ii) substrates which are present within the Loop region of a stem-loop LOCS reporter probe structures, and (iii) substrates which are present within the first oligonucleotide component of M-Tec-P probe complexes as described herein. Linear PlexZyme substrates which are dual labelled are known in the art and have been used for direct detection of nucleic acid sequences and/or for monitoring the accumulation of amplicons by various amplification methods. The cleavage of a linear substrate may separate a fluorophore and quencher allowing detection of a target over a broad range of temperatures. Linear substrates are known in the art as MNzyme substrates, MNzyme reporters, MNzyme probes, PlexZyme substrates, PlexZyme reporters, PlexZyme probes, Linear PlexZyme probes or standard PlexZyme probes. Cleavage of the Loop region of a LOCS by a PlexZyme may generate a Split LOCS structure composed of two fragment which may remain hybridized and associated at temperatures below the melting temperature of the stem and which may separate and dissociate at temperatures above the melting temperature of the stem of the split LOCS. The dissociation of split a LOCS substrate may separate a fluorophore and quencher allowing detection of a target at relatively high temperatures that are above the melting temperature of split stems but below the temperature of stems of intact LOCS. Cleavage of substrates which are present within the first oligonucleotide component of an Intact M-Tec-P probe by a PlexZyme may generate cleavage fragments of the first oligonucleotide component which are hybridized to a second oligonucleotide component at temperatures below the melting temperature of duplexes of the first capture region and second oligonucleotide component. At these temperatures, the second oligonucleotide component may also remain hybridized to the unmodified, intact first oligonucleotide component. At temperatures above the melting temperature of the complementary regions of the first capture region and second oligonucleotide component, or the capture region of first fragment of the first oligonucleotide component and second oligonucleotide component, the second oligonucleotide component may separate and dissociate. At temperatures

above the melting temperature of complementary regions of the first capture region and second oligonucleotide a constant level of signal will contribute a background level of fluorescence regardless of whether the first oligonucleotide component is intact or cleaved. As such, target dependent increases in fluorescence associated with target dependent cleavage can only be detected at lower temperatures below the melting temperature of the complementary regions of the first capture region and second oligonucleotide.

PlexZyme cleavage of a substrate may lead to separation of fluorophore and a quencher dye pair, which in turn, may generate a fluorescent signal. Cleavage of various types of PlexZyme substrates may result in changes in fluorescence above background which are observable over a broad range of temperatures, or at only specific temperatures. By way of example, cleavage of dual labelled linear PlexZyme substrates may generate fluorescence above background which may be monitored over a broad temperature range. In other examples, cleavage of substrates within the loop of a LOCS reporter probe may generate fluorescence above background which may only be monitored at temperatures which are above the melting temperature of the cleaved fragments of a Split LOCS structure. In other examples, cleavage of PlexZyme substrates present within the first oligonucleotide component of an M-Tec-P Probe may generate fluorescence above background which may be monitored at temperatures which are below the melting temperature of the complementary regions of capture region of the first component and second oligonucleotide components.

The terms “detectable effect” and “detectable signal” are used interchangeably herein and will be understood to have the same meaning. The terms refer to a signal or an effect generated from the detection moiety of a probe of the present invention (e.g. an oligonucleotide(s), reporter probe or substrate), typically upon modification of the probe to alter its conformation, structure, orientation, position relative to other entit(ies), and the like. The modification may, for example, be induced by the presence of a target that the probe is designed to detect. The detectable effect and detectable signal may be measurable only under specific conditions of measurement which may, or may not, be the same as conditions which can induce modification of the probe in the presence of the target. The detectable effect and detectable signal is a target dependent effect or signal which is measurable under the specific conditions of measurement for the probe which is designed to detect the specific target. The detectable effect or signal differs from background signal and may increase or decrease in magnitude compared to background levels. The change in detectable effect or signal compared to background signal or effect,

may be observable only in the presence of target, but not in the absence of target, under the specific conditions of measurement. The detectable effect or signal may comprise contributions generated from both target dependent modification of a first probe measurable at a defined temperature combined with any background signal generated from the first probe and/or other probes in the mixture. Non-limiting examples of such modifications (e.g. those induced by the presence of the target) include the opening of the stem-loop portion of a Molecular Beacon, the opening of double-stranded portion of Scorpion Uniprobos and Biprobos, the binding of Dual Hybridization Probes and Double-stranded probes (Yin-Yang probes) to a target sequence, the production of a Catcher-Pitcher Duplex, and cleavage/digestion of a linear PlexZyme substrate, M-Tec Probe, LOCS probe, or a TaqMan probe, and the like. The detectable signal may be detected by a variety of methods, including fluorescence spectroscopy, surface plasmon resonance (SPR), mass spectroscopy, NMR, electron spin resonance, polarization fluorescence spectroscopy, circular dichroism, immunoassay, chromatography, radiometry, photometry, scintigraphy, electronic methods, electrochemical methods, UV, visible light or infra-red spectroscopy, enzymatic methods or any combination thereof. The detectable signal/effect can be detected or quantified, and its magnitude may be indicative of the presence and/or quantity of an input such as the amount of a target molecule present in a sample. Further, the magnitude of the detectable signal/effect provided by the detection moiety may be modulated by altering the conditions of a reaction in which a probe comprising the detection moiety is utilised, including but not limited to, the reaction temperature. The capacity of the detection moieties attached or otherwise connected to the oligonucleotides to generate target-dependent signal, and/or target-independent background signal, can thus be modulated. A “detection moiety” may be a fluorophore or a quencher.

As used herein the terms “background signal”, “background level” and “baseline signal” are used interchangeably and will be understood to have the same meaning. The terms refer to signal generated by the detectable moiety of a first probe in the absence of the first specific target which the first probe is designed to measure or detect under the first specific conditions of measurement. Additional fluorescent signal generated by a detectable moiety of additional probes present in the reaction, which are not designed to measure or detect the first specific target under the first specific conditions of measurement, may also contribute to the “background signal” and “baseline signal”. The additional probes may be designed to detect additional target(s) under different conditions of measurement. The “background signal” and “baseline signal” generated under the first

specific conditions of measurement by the additional probes, may be independent of the presence or absence of the additional target(s) which the additional probes are designed to detect. The detection moieties on the first and additional probes may be the same, or may be different moieties which are measurable, for example, at the same wavelength.

As used herein the terms “background fluorescence” and “baseline fluorescence” are used interchangeably and will be understood to have the same meaning. The terms refer to fluorescent signal generated by the detectable moiety of a first probe in the absence of the first specific target which the first probe is designed to measure or detect under the first specific conditions of measurement. Additional fluorescent signal generated by a detectable moiety of additional probes present in the reaction, which are not designed to measure or detect the first specific target under the first specific conditions of measurement, may also contribute to the “background fluorescence” and “baseline fluorescence”. The additional probes may be designed to detect additional target(s) under different conditions of measurement. The “background fluorescence” and “baseline fluorescence” generated under the first specific conditions of measurement by the additional probes, may be independent of the presence or absence of the additional target(s) which the additional probes are designed to detect. The detection moieties on the first and additional probes may be the same, or may be different moieties which are measurable, for example, at the same wavelength.

The terms “polynucleotide substrate” and “oligonucleotide substrate” as used herein include any single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases, or analogues, derivatives, variants, fragments or combinations thereof, which is capable of being recognized, acted upon or modified by an enzyme including a catalytic nucleic acid enzyme. A “polynucleotide substrate” or “oligonucleotide substrate” may be modified by various enzymatic activities including but not limited to cleavage. Cleavage or degradation of a “polynucleotide substrate” or “oligonucleotide substrate” may provide a detectable effect for monitoring the catalytic activity of an enzyme. The “polynucleotide substrate” may be cleaved or degraded by one or more enzymes including, but not limited to, catalytic nucleic acid enzymes such as PlexZymes, AptaPlexZymes, DNAzymes, Aptazymes, ribozymes and/or protein enzymes such as exonucleases or endonucleases.

A “reporter substrate” as used herein is a substrate that is particularly adapted to facilitate measurement of either cleavage or degradation of a substrate or the appearance of a cleaved 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. Such labels may be referred to as “detectable moieties”.

As used herein, a “linear PlexZyme substrate” or “linear MNAzyme substrate” is a substrate, for example, a reporter substrate, that is recognized by and acted on catalytically by a plurality of PlexZymes. A “linear PlexZyme substrate” does not contain sequences at its 5’ or 3’ ends which are capable of hybridizing to form a stem. Alternatively, “PlexZyme substrates” may be present within the Loop region of a LOCS probe. In other examples, “PlexZyme substrates” may be present within the first oligonucleotide component of an M-Tec-P probe.

As used herein, a “universal substrate” is a substrate, for example, a reporter substrate, that is recognized by and acted on catalytically by a plurality of PlexZymes, 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 PlexZymes all of which recognize a universal substrate. Further, the same “universal substrate” sequence can be present within multiple probe types including “Linear PlexZyme substrate” and/or the loop region of a “LOCS probe” and/or in the first oligonucleotide component of an “M-Tec-P” probe complex. These universal substrates can each be independently labelled with one or more labels. In some 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 PlexZymes. In some embodiments the “universal substrates” may be capable of catalytic modification by DNazymes which are catalytically active in the presence of a cofactor, for example a metal ion co-factor such as lead or mercury. In some embodiments, the substrates may be amenable to catalytic modification by aptazymes which may become catalytically active in the presence of an analyte, protein, compound or molecule capable of binding to the aptamer portion of the aptazyme thereby activating the catalytic potential of the nucleic acid enzyme portion.

The terms “probe” and “reporter probe” as used herein refer to an oligonucleotide(s) or oligonucleotide complex that is used for detection of a target molecule (e.g. a nucleic acid or an analyte). Non-limiting examples of Standard Probes or Reporter probes, which are well known in the art include, but are not limited to, linear PlexZyme substrates, LOCS probes, TaqMan probes or hydrolysis probes, Molecular Beacons, Sloppy Beacons, Eclipse probes, Scorpion Uni-Probe, Scorpion Bi-Probes primer/probes,

catcher/pitcher oligonucleotides, Double-stranded probes (Yin-Yang probes) and dual-hybridization probes. Embodiments of the present invention combine standard probes with M-Tec Probes.

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” or “cleavage fragment” are used interchangeably to refer to a new molecule produced as a result of cleavage, degradation or endonuclease activity by an enzyme. In some embodiments, the products may be produced by enzymatic cleavage or degradation of the first oligonucleotide component of an M-Tec probe. In other embodiments, the products may be produced by enzymatic cleavage or degradation of an intact, LOCS structure comprise two oligonucleotide fragments, collectively referred to as a Split LOCS, wherein the two oligonucleotide fragments may be capable of either hybridization or dissociation/separation depending upon the temperature of the reaction.

As used herein, use of the terms “melting temperature” and “ T_m ” in the context of a polynucleotide will be understood to be a reference to the temperature at which half of two complementary strands of polynucleotide are in a single stranded state. The melting temperature (T_m) may be estimated using the Wallace rule, whereby $T_m = 2^{\circ}\text{C} (A+T) + 4^{\circ}\text{C} (G+C)$ (see Wallace *et al.*, (1979) *Nucleic Acids Res.* 6, 3543). The effects of sequence composition on the melting temperature can be understood using the nearest neighbour method, which is governed by the following formula: $T_m (^{\circ}\text{C}) = \Delta H^{\circ} / (\Delta S^{\circ} + R \ln[\text{oligo}]) - 273.15$. In addition to the length and sequence composition of fully or partially complementary regions of sequence, other factors that are known to impact the melting temperature include ionic strength, the presence of specific solvents and oligonucleotide concentration. A higher oligonucleotide and/or ion concentration increases the chance of duplex formation which leads to an increase in melting temperature. In contrast, a lower oligonucleotide and/or ion concentration favours dissociation of the stem which leads to a decrease in melting temperature. In some nonlimiting embodiments, when the defined temperature of measurement of a fluorescence associated with an M-Tec probe is selected to be, for example, below the T_m of the duplex formed by the first and second oligonucleotide components, the temperature may be chosen such that the majority of oligonucleotide components are double stranded. Alternatively, when defining the temperature for measuring fluorescence generated by an M-Tec probe, for example, below the T_m of the duplex formed by the first capture region and second oligonucleotide components, the temperature may be chosen such that the majority of first capture regions and second oligonucleotide components are double

stranded. Similarly, when defining the temperature for measuring fluorescence generated by a LOCS probe, for example, above the T_m of the duplex formed by the two fragments of a cleaved LOCS probe, the temperature may be chosen such that the majority of fragments are single stranded. A suitable measurement temperature for detecting cleaved LOCS probes may also be below the T_m of the duplex formed by the two stems of an intact LOCS probe, the temperature is chosen such that the majority of intact LOCS probes are in a hair-pinned conformation.

As used herein the term “quencher” includes any molecule that when in close proximity to a fluorophore, takes up emission energy generated by the fluorophore and either dissipates the energy as heat or emits light of a longer wavelength than the emission wavelength of the fluorophore. Non-limiting examples of quenchers include Dabcyl, TAMRA, graphene, FRET fluorophores, ZEN quenchers, ATTO quenchers, Black Hole Quenchers (BHQ), Iowa Black Dark Quenchers and Black Berry Quenchers (BBQ).

A “fluorophore” is a photoreactive molecule that can emit light upon excitation. A fluorophore may be directly attached to another molecule such as an oligonucleotide or a protein where it serves as a tag or label. A fluorophore may be directly attached to an oligonucleotide, forming a probe which may be used to detect a specific target. Fluorophores or fluorophore moieties may also be referred to as “reporter moieties” or “reporter labels”. Different fluorophores may emit at different wavelengths, or they may emit at the same or at a similar wavelength. Non-limiting examples of fluorophores include fluorescein, Texas red, Alexa Fluor 350, BODIPY FL, Oregon Green 488, SUN fluorophore and ATTO 647N. Fluorophores and quenchers are examples of detection moieties which may be directly attached to oligonucleotides useful as reporter probes.

As used herein, the term “base” when used in the context of a nucleic acid will be understood to have the same meaning as the term “nucleotide”.

As used herein the term “blocker” or “blocker molecule” refers to any molecule or functional group which can be incorporated into an oligonucleotide to prevent a polymerase using a portion of the oligonucleotide as a template for the synthesis of a complementary strand. By way of a non-limiting example, a hexathylene glycol blocker can be incorporated into, for example, a Scorpion probe to link its 5' probing sequence to its 3' priming sequence, wherein the blocker functions to prevent a polymerase using the probing sequence as a template.

As used herein the terms “normalise”, “normalising” and “normalised”, refer to the conversion of a measured signal (e.g. a detectable signal generated by a detection moiety) to a scale relative to a known and repeatable value or to a control value.

As used herein, the term “kit” refers to any delivery system for delivering materials. Such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (for example labels, reference samples, supporting material, etc. in the appropriate containers) and/or supporting materials (for example, buffers, written instructions for performing an assay etc.) from one location to another. For example, kits may include one or more enclosures, such as boxes, containing the relevant reaction reagents and/or supporting materials. The term “kit” includes both fragmented and combined kits.

As used herein, the term “fragmented kit” refers to a delivery system comprising two or more separate containers that each contains a subportion of the total kit components. The containers may be delivered to the intended recipient together or separately. Any delivery system comprising two or more separate containers that each contains a subportion of the total kit components are included within the meaning of the term “fragmented kit”.

As used herein, a “combined kit” refers to a delivery system containing all of the components of a reaction assay in a single container (e.g. in a single box housing each of the desired components).

It will be understood that use 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.

It will be understood that 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 polypeptide of between 10 residues and 20 residues in length is inclusive of a polypeptide of 10 residues in length and a polypeptide of 20 residues in length.

Any description of prior art documents herein, or statements herein derived from or based on those documents, is not an admission that the documents or derived statements are part of the common general knowledge of the relevant art.

For the purposes of description all documents referred to herein are hereby incorporated by reference in their entirety unless otherwise stated.

Abbreviations

The following abbreviations are used herein and throughout the specification:

M-Tec Probe: Multiple-component Temperature Controlled Probe;
M-Tec-P Probe: M-Tec Probe suitable for cleavage by a catalytic nucleic acid such as a PlexZyme;
M-Tec-H Probe: M-Tec Probe suitable for hydrolysis by an exonuclease;
M-Tec-E Probe: M-Tec Probe suitable for cleavage by an endonuclease;
OC1: First oligonucleotide component of an M-Tec Probe;
OC2: Second oligonucleotide component of an M-Tec Probe;
OC3: Third oligonucleotide component of an M-Tec Probe;
T_m OC1/OC2: melting temperature of complementary regions of OC1 and OC2;
T_m OC1/OC3: melting temperature of complementary regions of OC1 and OC3;
LOCS: loop connected to stems;
MNAzyme (also called PlexZyme): multi-component nucleic acid enzyme;
Partzyme: Partial enzyme containing oligonucleotide;
PCR: polymerase chain reaction;
gDNA: genomic DNA;
NTC: No template control;
qPCR: Real-time quantitative PCR;
C_t: Threshold cycle;
C_q: Quantification cycle;
R²: Correlation coefficient;
nM: Nanomolar;
mM: Millimolar;
pM: Picomolar;
μL: Microlitre;
μM: Micromolar;
dNTP: Deoxyribonucleotide triphosphate;
NF-H₂O: nuclease-free water;
LNA: locked nucleic acid;
F: fluorophore;
Q: quencher;
N = A, C, T, 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;

R: A, G, or AA;

rN: any ribonucleotide base;

(rN)_x: any number of rN;

rR: A or G;

rY: C or U;

M: A or C;

H: A, C, or T;

D: G, A, or T;

JOE or 6-JOE: 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein;

FAM or 6-FAM: 6-Carboxyfluorescein;

HEX: Hexachlorofluorescein;

SUN: SUN™ fluorophore;

ATTO647N: ATTO 647N (NHS-ester);

BHQ1: Black Hole Quencher 1;

BHQ2: Black Hole Quencher 2;

Phos: Phosphorylation

RT-PCR: reverse transcription polymerase chain reaction ;

SDA: strand displacement amplification;

NEAR: Nicking Enzyme Amplification Reaction;

HDA: helicase dependent amplification;

RPA: Recombinase Polymerase Amplification;

LAMP: loop-mediated isothermal amplification;

RCA: rolling circle amplification;

TMA: transcription-mediated amplification;

3SR: self-sustained sequence replication;

NASBA: nucleic acid sequence based amplification;

LCR: Ligase Chain Reaction;

RAM: Ramification Amplification Method;

IB: Iowa Black® FQ;

IBR: Iowa Black® RQ;

shRNA: short hairpin RNA;

siRNA: short interfering RNA;

mRNA: messenger RNA;

tRNA: transfer RNA;

snoRNA: small nucleolar RNA;

stRNA: small temporal RNA;
smRNA: small modulatory RNA;
pre-microRNA: precursor microRNA;
pri-microRNA: primary microRNA;
LHS: Left hand side;
RHS: Right hand side;
DSO: double stranded oligonucleotide;
T_m: Melting Temperature;
RFU: Relative Fluorescence Units;
CT: Chlamydia trachomatis;
NG: Neisseria gonorrhoeae;
SPR: surface plasmon resonance;
GNP: gold nanoparticles;
DNS: duplex specific nuclease;

Detailed Description

The following detailed description conveys exemplary embodiments of the present invention in sufficient detail to enable those of ordinary skill in the art to practice the present invention. Features or limitations of the various embodiments described do not necessarily limit other embodiments of the present invention or the present invention as a whole. Hence, the following detailed description does not limit the scope of the present invention, which is defined only by the claims.

The present invention relates to methods and compositions for the multiplexed detection of one or more targets (e.g. nucleic acids, proteins, analytes, compounds, molecules and the like). The methods and compositions each employ a combination of oligonucleotide complexes herein referred to as M-Tec Probes optionally used together with other oligonucleotide reporters, probes or substrates, which may further be used in combination with various other agent/s.

- *Probes and Substrates*

According to the present invention, multiplex detection of target molecules is facilitated using M-Tec Probes in combination with another nucleic acid suitable for use as a probe in a multiplex detection assay.

Many nucleic acid probes for detection of nucleic acid targets have been described and are well known in the art. Suitable nucleic acid probes that can be used in combination with M-Tec Probes include, but are not limited to, LOCS probes, linear PlexZyme substrates, TaqMan or Hydrolysis probes, Molecular Beacons, Sloppy Beacons, Eclipse probes, Amplifluor/Sunrise primer probes, Scorpion Uni-Probe, Scorpion Bi-Probes, dual-hybridization probes, Double-stranded probes, (Yin-Yang probes) and Catcher/Pitcher probes

In some embodiments, these nucleic acid probes bind directly to the target or target amplicon to facilitate their detection, however, probe types that incorporate PlexZyme substrates, and Catcher oligonucleotides for TOCE, provide an exception as they may be universal and suitable for detection of any target.

In some embodiments, the nucleic acid probes generate fluorescence in the presence of target due to enzymatically mediated cleavage or degradation, for example, M-Tec Probes, LOCS Probes, linear PlexZyme substrates and TaqMan or Hydrolysis probes.

In other embodiments, the nucleic acid probes provide different levels of fluorescent signal as a result of a conformation change induced by binding to a target or target amplicon (e.g. Molecular Beacons, Sloppy Beacons, Eclipse probes, Scorpion Uni-Probe, Scorpion Bi-Probes, Double-stranded probes (Yin-Yang probes) and dual-hybridization probes).

In the TOCE system, the Catcher changes fluorescence as a result of conformation changes induced by binding and extension of the Pitcher which is only activated and released in the presence of target.

Any, or all, of these types of reporter nucleic acid probes are suitable for use in conjunction with M-Tec Probes to mediate detection of multiple targets by measurement of changes related to a single detection moiety, including but not limited to, a change in fluorescence measured at a single wavelength. It is also possible to combine M-Tec probes with other probe types whereby the probes are labelled with different fluorophores which have the same or similar emission spectra and hence are measurable at the same wavelength.

Oligonucleotides for M-Tec Probes or for probes used in combination with M-Tec Probes can be synthesised according to standard protocols. For example, they may be synthesised by phosphoramidite chemistry, using nucleoside and non-nucleoside phosphoramidites in sequential synthetic cycles that involves removal of the protective group, coupling the phosphoramidites, capping and oxidation, either in solid-phase or solution-phase and optionally in an automated synthesiser device. Alternatively, they may

be purchased from commercial sources. Non-limiting examples of commercial sources from which linear or LOCS PlexZyme substrates, TaqMan or Hydrolysis probes, Molecular Beacons, Sloppy Beacons, Eclipse probes, Scorpion Uni-Probe, Scorpion Bi-Probes, dual-hybridization probes, Double-stranded probes (Yin-Yang probes) and Catcher/Pitcher probes can be purchased or otherwise obtained include: PlexZyme substrates can be purchased from SpeedX (plexpcr.com); TaqMan and hydrolysis probes can be purchased from Thermo Fisher Scientific (www.thermofisher.com), Sigma Aldrich (www.sigmaaldrich.com), Promega (www.promega.com), Geni Biotech (www.generi-biotech.com); Molecular Beacons and Sloppy beacons may be purchased from Integrated DNA Technologies (www.idtdna.com), Eurofins (www.eurofinsgenomics.com) and TriLink BioTechnologies (www.trilinkbiotech.com); Eclipse probes can be purchased from Integrated DNA Technologies (www.idtdna.com); Scorpion Uni-Probes can be purchased from Sigma Aldrich (www.sigmaaldrich.com) and Bio-Synthesis (<https://www.biosyn.com>); Scorpion bi-probes can be purchased from Bio-Synthesis (<https://www.biosyn.com>); Dual-hybridisation probes can be purchased from Bio-Synthesis (<https://www.biosyn.com>), Sigma Aldrich (www.sigmaaldrich.com) and Eurofins (www.eurofinsgenomics.com); Double-stranded probes (Yin-Yang probes) can be purchased from Integrated DNA Technologies (www.idtdna.com); and Catcher Pitcher assays may be purchased from Seegene (www.seegene.com).

- ***M-Tec Probe Oligonucleotides***

Provided herein are methods and compositions which extend the capacity for multiplex analysis of nucleic acid targets. These methods and compositions employ Multiple-component Temperature-controlled Probes herein referred to as M-Tec Probes. By way of example, M-Tec Probes are multiple-component complexes comprising at least two oligonucleotide components wherein a first oligonucleotide component (OC1) is connected to a first detection moiety and is capable of being modified by enzymatic activity in the presence of a target, and a second oligonucleotide component (OC2) is labelled with a second detection moiety. The first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a double-stranded portion. The first and second oligonucleotide components are capable of hybridization at temperatures below the melting temperature (T_m) of the double-stranded portion (T_m OC1/OC2). The first oligonucleotide component is connected to a detection moiety either by direct labelling, or via a region of complementarity with a third oligonucleotide component (OC3) which

is directly labelled with the detection moiety. If present, a third oligonucleotide component is capable of hybridization with the first oligonucleotide component at temperatures below the T_m of the complementary regions (T_m OC1/OC3). If this temperature is greater than the T_m OC1/OC2, then at temperatures below T_m OC1/OC2 all oligonucleotide components in a complex will be hybridized. The first and second detection moiety may be, for example, a fluorophore and a quencher or vice versa. The region or position which is subject to enzymatic modification is located between the first capture region and the first detection moiety. In some embodiments, an M-Tec Probe only comprises a first and a second detection moiety and no additional detection moieties. In some embodiments, the first and second detection moieties are located on different oligonucleotides, namely the first and second oligonucleotide components, neither of which is dual labelled.

When all oligonucleotide components are hybridized, and the first oligonucleotide component is unmodified, the fluorophore and quencher are in close proximity resulting in an intact M-Tec Probe complex which is quenched. In the presence of a target, a sensor region of the first oligonucleotide component is modified, for example by cleavage or hydrolysis by an enzyme. Enzymatic modification of the sensor region of the first oligonucleotide component generates a first fragment comprising the first capture region connected to the second detection moiety and a second fragment connected to the first detection moiety, thereby enabling the first and second detection moieties to spatially separate and generate a first detectable signal. By way of example, this modification occurs in a sensor region positioned between the two detection moieties such that it causes separation of, for example, the fluorophore and the quencher. Resultant target-dependent increase in fluorescence can be measured at temperatures below T_m OC1/OC2. At temperatures above the T_m OC1/OC2, the first and second oligonucleotide components dissociate, and the detection moieties are separated, resulting in constant contribution to the background levels of fluorescence regardless of the presence or absence of target. At this temperature the second oligonucleotide component will no longer hybridize to either the unmodified first oligonucleotide component in reactions where no target is present, or to a hydrolysed/cleaved fragment of the first oligonucleotide component, which has been modified in the presence of target. In this manner M-Tec Probes will generate target dependent increases in fluorescence at temperatures below T_m OC1/OC2 but no change in fluorescence will be observed regardless of the presence or absence of target at temperatures above the T_m OC1/OC2.

Various types of M-Tec Probes are disclosed and exemplified (see, e.g., **Figures 1, 16 to 19**). Although exemplified with a fluorophore/quencher pair, the skilled addressee will recognise that any other suitable detection moieties may be used for the same purpose. One type of M-Tec Probe is suitable for modification/cleavage by an PlexZyme in the presence of target. These probes are herein referred to as M-Tec-P probes, exemplary components and complexes are illustrated in Figure 1(i) and Figures 2, 3 and 17. An M-Tec-P may have two oligonucleotide components, namely a first oligonucleotide component (OC1) directly labelled with a detection moiety, for example a quencher, and comprising a sensor region that serves as a substrate for a PlexZyme, and a second oligonucleotide component (OC2) directly labelled with a second detection moiety, for example a fluorophore (**Figures 1 (i) and 2(ii)**). Alternatively, M-Tec-P complexes may be labelled with a fluorophore at or near one terminus and comprise a sensor region that serves as a substrate for a PlexZyme, and an OC2 labelled with a quencher (**Figure 1(ii)**). The OC1 comprises a first capture region capable of hybridisation to OC2 at “low” temperatures below the melting temperature of their complementary regions (T_m OC1/OC2) (**Figures 3(i) and ii**). The site amenable to modification/cleavage by the PlexZyme may be located within the sensor region between the capture region and the first detection moiety.

M-Tec-P probes may be designed to be cleavable by PlexZymes which can assemble from component partzymes, when the partzymes bind adjacently to complementary regions on the target to be detected (**Figures 1-3**). The M-Tec-P may then bind to the PlexZyme following hybridization of the OC1 with the substrate binding arms of a PlexZyme. (**Figure 1(i) and (iii) and Figure 2(iv)**). In some embodiments, an OC2 hybridizes to a capture region of the OC1 which does not hybridize/bind to the substrate binding arms of the partzymes as illustrated in Figure (**Figure 1(i) and (iii) and Figure 2(iv)**). Cleavage of the sensor region of the OC1 of the M-Tec-P generates two OC1 fragments, one of which is connected to the quencher and the other contains the capture region complementary to the OC2 (**Figure 3(i) and (iii)**). At “low” temperatures below the T_m OC1/OC2, an increase in fluorescence is indicative of the presence of the target which facilitated assembly of the PlexZyme (**Figure 3(i) and Figure 4 top middle panel**). In the absence of target, M-Tec probes are not cleaved and the fluorophore and quencher are not separated and there is no increase in fluorescence above background during the reaction at this temperature (**Figure 3(ii) and Figure 4 top middle panel**). At “high” temperatures above the T_m OC1/OC2, no change in fluorescence is observed regardless of the presence or absence of the target (**Figure 3(iii) and (iv) and Figure 4**

bottom middle panel). Background or baseline fluorescence resulting from dissociation of OC1 and OC2 is constant and is not affected by the presence or absence of target.

In some embodiments the M-Tec-P probe may be universal. A universal M-Tec-P structure may contain a universal first component oligonucleotide comprising a universal first capture region, and a universal sensor region which includes a universal catalytic nucleic acid substrate which can be cleaved by any PlexZyme with complementary substrate binding arms regardless of the sequences of the PlexZyme target sensing arms. A single universal M-Tec-P probe can be used as a surrogate marker for any target which is capable of facilitating the cleavage of a specific M-Tec-P probe. A series of universal M-Tec-P probes can be incorporated into any multiplex assay designed to analyse any set of targets. Additionally, the universal first oligonucleotide component may be connected to a first detection moiety which may be a quencher moiety that has the capability of quenching a range of different fluorophores. In such cases the first universal capture region of the first oligonucleotide component may be capable of hybridization to any of a series of second oligonucleotide components, each of which has the same sequence but is labelled with a different fluorophore that may be quenched by the quencher connected to the universal first oligonucleotide component. Some M-Tec-P probes comprise nucleic acid enzyme substrates within the loop regions which may be universal, and which are capable of catalytic cleavage by nucleic acid enzymes such as PlexZymes, DNAzymes, aptazymes and ribozymes. By way of example, the same universal M-Tec-Probe may be used in conjunction with many target specific PlexZymes to detect a range of nucleic acid targets and further may be used in conjunction with many target specific aptazymes to detect a range non-nucleic acid targets.

Another type of M-Tec Probe is suitable for hydrolysis by exonuclease activity, for example, by Taq polymerase in the presence of target. These probes are herein referred to as M-Tec-H probes and exemplary components and complexes are illustrated in **Figure 1(ii) and (iv)** and **Figure 7**. An M-Tec-H may have two oligonucleotide components, the OC1 and OC2. The OC1 may be labelled with a quencher at or near one terminus and have a sensor region, of which at least a portion is complementary to the target to be detected; whilst the OC2 may be labelled with a fluorophore (**Figures 1 (ii) and 7**). Alternatively, M-Tec-H complexes may comprise an OC1 labelled with a fluorophore at or near one terminus, and an OC2 labelled with a quencher (**Figure 1 (iv)**). OC1 and OC2 have regions of complementary and are capable of hybridization or association at “low” temperatures below the melting temperature of their complementary regions ($<T_m$

OC1/OC2) (**Figures 7(i) and ii**). In some embodiments, the OC2 hybridizes to a capture region of the OC1 which does not hybridize/bind to the target.

M-Tec-H probes are designed to be hydrolysed by exonuclease activity in the presence of target. During PCR, at least a portion of the sensor region of the OC1 of the M-Tec-H may bind to the target amplicon and at least a portion of the sensor region may be hydrolysed by the exonuclease activity of polymerase. Hydrolysis of the OC1 of the M-Tec-H Probe results in generation of two OC1 fragments, one of which is connected to the first detection moiety, for example a quencher and the other of which contains the capture region of complementarity with the OC2 (**Figure 7(i) and (iii)**). At “low” temperatures below the T_m OC1/OC2, an increase in fluorescence is indicative of the presence of the target (**Figure 7(i)**). In the absence of target, M-Tec-H probes are not hydrolysed and there is no increase in fluorescence above background during the reaction (**Figure 7(ii)**) at this temperature. At “high” temperatures above the T_m OC1/OC2, no change in fluorescence is observed regardless of the presence or absence of the target (**Figures 7(iii) and (iv)**). Background fluorescence contributed from dissociation of OC1 and OC2 is constant and is not affected by the presence or absence of target.

In some embodiments, M-Tec Probes are used in combination with hairpin universal PlexZyme probes (**Figure 6** and **Figure 8**), also known as LOCS (Loops Connected to Stems) Probes (**Figure 9** and **Figure 10**). In other embodiments M-Tec Probes can be combined with other Probe and Substrate types known in the art, including, but not limited to, linear PlexZyme substrates, standard dual-labelled TaqMan probes or Hydrolysis probes, Molecular Beacons, Sloppy Beacons, Eclipse probes, Scorpion Uni-Probes or Bi-Probes, Catcher-Pitcher Oligonucleotides, Double-stranded probes (Yin-Yang probes), and dual-hybridization probes.

The combination of M-Tec Probes with other Probe or Substrate types allows greater multiplexing capacity, wherein multiple targets can be detected, identified and/or qualified at a single wavelength. By way of example, an M-Tec Probe, together with one or more LOCS probes, both of which incorporate the same detection moiety (e.g. the same fluorophore) can be used to individually discriminate multiple targets within a single reaction. Alternatively, multiple targets may be discriminated within a single reaction using an M-Tec Probe and a LOCS probe, which incorporate different detection moieties that emit signal at a similar wavelength and that may be monitored in the same fluorescent channel on a detection instrument. The approach involves measurement of the signal generated from the probes at discrete temperatures. In some embodiments, a first target is measured at a first temperature by monitoring changes in fluorescence associated

with modification of an M-Tec Probe and a second target is measured at a second temperature by monitoring changes in fluorescence associated with modification of a LOCS probe (**Figures 6 and 8**).

The properties of the M-Tec probe components, and other probe types with which they may be combined to increase multiplexing capacity, is part of the design of the multiplexed assay. In particular, the T_m of regions of complementarity between components of probes (intermolecular bonds), or within probes (intramolecular bond) and between components or probes and the targets (intermolecular bonds) influences the capacity to combine various probe types. Exploitation of these properties of the probes and/or their components, allows manipulation of the association or dissociation of regions of probes, and probe complex components, at defined temperatures. In turn, these properties influence whether or not various probe types generate target specific fluorescence at specific acquisition temperatures or whether only background fluorescence is observed in the presence or absence of target. This may be better understood by consideration of the scenarios for different probe types tabulated in Tables 1, 2 and 3 below. The scenarios are exemplary only and one skilled in the art will appreciate that there are many other scenarios that can be envisaged for combining probes to allow analysis of multiple targets at a single wavelength when the principles of manipulation of target dependent fluorescence and background outlined above and below are applied.

Table 1: Relation of T_m of components at acquisition Temperature 1 (Temp 1) and acquisition Temperature 2 (Temp 2) in the presence of target (+ T) or in the absence of target (-T), Scenarios which result in increased detectable signal which is measured as Fluorescence above background (F) or which contribute to the Background Signal only (B) at Temp 1 and 2 are tabulated.

Probe Type	Interrelationship between the T _m 's of probes and components and Temp 1 and 2	Temp 1	Temp 2
M-Tec Probe	T _m OC1/OC2 > Temp 1 T _m OC1/OC2 < Temp 2	+ T = F - T = B	+/- T = B
LOCS probe	Intact T _m Stem > Temp 2 Split T _m Stem > Temp 1 Split T _m Stem < Temp 2	+/- T = B	+ T = F - T = B
Molecular Beacon	T _m Stem < T _m Loop/Target T _m Stem and T _m Loop/Target > Temp 1 T _m Stem and T _m Loop/Target < Temp 2	+ T = F - T = B	+/- T = B
Linear PlexZyme probe	Not Applicable	+ T = F - T = B	+ T = F - T = B
TOCE Probe	T _m Catcher/Pitcher > Temp 1 T _m Catcher/Pitcher < Temp 2	+ T = F - T = B	+/- T = B
	T _m Catcher/Pitcher > Temp 1 T _m Catcher/Pitcher > Temp 2	+ T = F - T = B	+ T = F - T = B

As such, the following features can be used to manipulate generation of target dependent fluorescence at specific acquisition temperatures for various probe types. By way of example, when the components of M-Tec probes have a T_m OC1/OC2 which is above a first acquisition temperature but below the second acquisition temperature, target dependent fluorescence will be observed only at the first acquisition temperature. Background fluorescence only will be observed at the first temperature in the absence of target, and background fluorescence only will be observed at the second temperature regardless of the presence or absence of target.

When the T_m of the stem of an intact/uncleaved LOCS probe is above the second acquisition temperature, and the T_m of the stem of a cleaved split LOCS probe is above the first acquisition temperature but below the second acquisition temperature, target dependent fluorescence will be observed only at the second acquisition temperature. Background fluorescence only will be observed at the second temperature in the absence

of target and background fluorescence only will be observed at the first temperature regardless of the presence or absence of the LOCS probe target.

When the T_m of the stem of a Molecular Beacon is less than the T_m of the loop/target hybrid, and the T_m of both the stem and the loop/target hybrid are above the first acquisition temperature but below the second acquisition temperature, target dependent fluorescence will be observed only at the first acquisition temperature. Background fluorescence only will be observed at the first temperature in the absence of target and background fluorescence only will be observed at the second temperature regardless of the presence or absence of the molecular beacon's target.

Linear PlexZyme probes which have been cleaved in the presence of a target do not have the same capacity to have the fluorescence controlled by temperature. As such, once these probes are cleaved in the presence of target, they will fluoresce at both the first and second acquisition temperatures. These probes will produce background signal at both temperatures only when no target is present.

Catcher-Pitcher complexes have some capacity to have the target-dependent fluorescence controlled by temperature. In the presence of a target, the TOCE system produces double stranded Catcher-Pitcher complexes specific for each target. These complexes can be designed to be of any length and hence have T_m 's at various temperatures which are set at the designer's discretion. If the T_m of the Catcher-Pitcher complex is above the first acquisition temperature but below the second acquisition temperature, target dependent fluorescence will be observed only at the first acquisition temperature. Background fluorescence only will be observed at the first temperature in the absence of target and at the second temperature regardless of the presence or absence of target. If the T_m of the Capture/Pitcher complex is above both the first and second acquisition temperatures, target dependent fluorescence will be observed at both the first and second acquisition temperatures. Background fluorescence only will be observed at the first and second temperatures in the absence of target.

The capacity to manipulate target dependent fluorescence and background at various temperatures for specific probe types, as exemplified above, provides a broad general approach for designing systems for combining probe types that allow detection of targets at specific temperatures only. Further, it provides a wide range of options for detecting multiple targets at a single wavelength. This may be illustrated by non-exhaustive exemplary combinations tabulated in **Table 2**.

Table 2: Generation of a detectable signal measurable as Fluorescence (F) above background, or signals which contribute to Background only (B) when probe types are combined to detect Target 1 (T1) and Target 2 (T2) at a single wavelength under temperature relationships consistent with those defined in Table 1 and, where indicated, as further defined within Table 2. The presence and absence of Target are indicated as plus (+) or minus (–) respectively. Each pair of probe types in the table can be labelled with the same fluorophore and read at the same wavelength. Alternatively, each pair of probe types in the table can be labelled with different fluorophores provided the fluorophores have similar wavelengths which can be read in the same fluorescent channel. The total background signal at a given temperature may be the sum of background contributed from a probe in the absence of target plus from the background contributed additional probes in the presence or absence of target.

Probes (Target detected)	Temp 1	Temp 2	Analysis
M-Tec Probe (T1) LOCS Probe (T2)	+ T1 = F – T1 = B +/- T2 = B	+/- T1 = B – T2 = B + T2 = F	T1 = F at Temp 1 T2 = F at Temp 2
M-Tec Probe (T1) Linear PlexZyme probe (T2)	+ T1 = F + T2 = F – T1/T2 = B	+/- T1 = B + T2 = F – T2 = B	T1 = F at Temp 1 minus F at Temp 2 T2 = F at Temp 2
M-Tec Probe (T1) TOCE probe (T2) with Tm Catcher/Pitcher >Temp 2	+ T1 = F + T2 = F – T1/T2 = B	+/- T1 = B + T2 = F – T2 = B	T1 = F at Temp 1 minus F at Temp 2 T2 = F at Temp 2
Molecular Beacon (T1) LOCS Probe (T2)	+ T1 = F – T1 = B +/- T2 = B	+/- T1 = B – T2 = B + T2 = F	T1 = F at Temp 1 T2 = F at Temp 2
M-Tec Probe 1 (T1) M-Tec Probe 2 (T2) wherein Probe 1 Tm OC1/OC2 is < Probe 2 Tm OC1/OC2 and Probe 2 Tm OC1/OC2 < Temp 2	+ T1 = F + T2 = F – T1/T2 = B	+/- T1 = B + T2 = F – T2 = B	T1 = F at Temp 1 minus F at Temp 2 T2 = F at Temp 2

As exemplified in **Table 2**, there are numerous ways in which specific probe types can be combined to analyse multiple targets at a single wavelength. The relationships between the Tm of intramolecular bonds for each probe type, and for intermolecular

bonds between probe components and probes and targets, as exemplified in Table 1, underpin the capacity to multiplex different probe types for this purpose when signal is acquired at specific temperatures. By way of example, an M-Tec Probe designed to detect Target 1 can be combined with a LOCS probe designed to detect Target 2. In this scenario an increase in fluorescence above background will be observed for Target 1 at the first acquisition temperature only and an increase in fluorescence above background will be observed for Target 2 at the second acquisition temperature only. Conversely, background signal only will be observed at the first acquisition temperature in the absence of Target 1 regardless of the presence or absence of Target 2 and background signal only will be observed at the second acquisition temperature in the absence of Target 2 regardless of the presence or absence of Target 1. As such the presence of Target 1, and/or determination of the number of copies, can be determined by analysis of data acquired at temperature 1 and the presence of Target 2, and/or determination of the number of copies, can be determined by analysis of data acquired at temperature 2. In this scenario, the detectable signal generated in the presence of each specific target is discretely measured at a specific temperature and hence there is no requirement for subtraction of any detectable signal generated by the presence of other target(s) that may or may not be present in the reaction, since these will not be measurable at the same specific temperature. This provides a significant advantage over protocols which require algorithms to subtract target-dependent signal related to one target when multiple targets generate fluorescence at the same specific temperature of measurement.

An M-Tec Probe designed to detect Target 1 can be combined with a Linear PlexZyme probe designed to detect Target 2. In this scenario, an increase in fluorescence above background will be observed in the presence of either or both Target 1 and Target 2 at the first acquisition temperature, and an increase in fluorescence above background will be observed only in the presence of Target 2 at the second acquisition temperature. A background signal only will be observed at the first acquisition temperature in the absence of both Target 1 and Target 2. Background signal only will be observed at the second acquisition temperature in the absence of Target 2 regardless of the presence or absence of Target 1. As such the presence of Target 1, and/or determination of the number of -copies of Target 1, can be determined by analysis of data acquired at temperature 1 and temperature 2, where the contribution to fluorescence generated from target 1 can be calculated by subtracting the fluorescence acquired at temperature 2 from the total fluorescence acquired at temperature 1. The presence of Target 2, and/or determination of

the number of target copies, can be determined by analysis of data acquired at temperature 2 only.

An M-Tec Probe designed to detect Target 1 can be combined with a TOCE probe designed to detect Target 2 where the T_m of the Catcher/Pitcher complex is above acquisition temperature 2. In this scenario, an increase in fluorescence above background will be observed in the presence of either or both Target 1 and Target 2 at the first acquisition temperature, and an increase in fluorescence above background will be observed only in the presence of Target 2 at the second acquisition temperature. A background signal only will be observed at the first acquisition temperature in the absence of both Target 1 and Target 2. Background signal only will be observed at the second acquisition temperature in the absence of Target 2 regardless of the presence or absence of Target 1. As such the presence of Target 1, and/or determination of the number of target copies, can be determined by analysis of data acquired at temperature 1 and temperature 2, where the contribution to fluorescence from target 1 can be calculated by subtracting the fluorescence acquired at temperature 2 from the total fluorescence acquired at temperature 1. The presence of Target 2, and/or determination of the number of target copies, can be determined by analysis of data acquired at temperature 2 only.

A Molecular Beacon designed to detect Target 1 can be combined with a LOCS probe designed to detect Target 2. In this scenario, an increase in fluorescence above background will be observed for Target 1 at the first acquisition temperature only and an increase in fluorescence above background will be observed for Target 2 at the second acquisition temperature only. Conversely, background signal only will be observed at the first acquisition temperature in the absence of Target 1 regardless of the presence or absence of Target 2 and background signal only will be observed at the second acquisition temperature in the absence of Target 2 regardless of the presence or absence of Target 1. As such the presence of Target 1, and/or determination of the number of target copies, can be determined by analysis of data acquired at temperature 1 and the presence of Target 2, and/or determination of the number of target copies, can be determined by analysis of data acquired at temperature 2.

One skilled in the art will appreciate that there are many other scenarios that can be envisaged for combining probes to allow analysis of multiple targets at a single wavelength when the principles of manipulation of target dependent fluorescence and background outlined above are applied.

Table 2 gives multiple options for combinations of various probe types which incorporate the principle described in **Table 1**. It is possible to combine these further by

using different combinations of different probes labelled with the same fluorophores to be read in separate channels. This may be further understood by the non-exhaustive scenarios tabulated in **Table 3**.

Table 3: Exemplary reaction formats wherein multiple probe types, labelled with multiple Fluorophores, for example F1, F2 or F3, are combined to develop highly multiplex reactions whereby one or more Targets, for example T1, T2, T3, T4, T5 are measured by a single Fluorophore with acquisition of fluorescence at multiple temperatures, for example Temp 1 and Temp 2. When relationship of the T_m of component with respect to each other, and the acquisition temperature are as defined in Tables 1 and 2 then detectable signal measurable as Fluorescence (F) above background, or signals which contribute to the Background only (B), could be measured as tabulated below. The total background signal at a given temperature may be the sum of background contributed from a probe in the absence of target plus from the background contributed by additional probes in the presence or absence of target.

Fluorophore	Probes (Target detected)	Temp 1	Temp 2
F1	M-Tec Probe (T1)	+ T1 = F	+/- T1 = B
	LOCS Probe (T2)	- T1 = B	- T2 = B
		+/- T2 = B	+ T2 = F
F2	Molecular Beacon (T3)	+ T3 = F	+/- T3 = B
	LOCS Probe (T4)	- T3 = B	- T4 = B
		+/- T4 = B	+ T4 = F
F3	Linear PlexZyme Probe (T5)	+ T5 = F	+ T5 = F
		- T5 = B	- T5 = B

The scenarios outlined in **Table 3** provide an exemplary strategy which would allow for the detection, specific identification and/or quantification of five targets present in a single reaction when fluorescence is acquired at two temperatures at three different wavelengths specific for three fluorophores. At a first wavelength, specific for a first fluorophore, a first target could be monitored at a first acquisition temperature using an M-Tec probe specific for Target 1 and a second target could be monitored at a second acquisition temperature using a LOCS probe specific for Target 2. At a second wavelength, specific for a second fluorophore, a third target could be monitored at a first acquisition temperature using a Molecular Beacon specific for Target 3 and a fourth target could be monitored at a second acquisition temperature using a LOCS probe specific for

Target 4. At a third wavelength, specific for a third fluorophore, a fifth target could be monitored at either the first and/or the second acquisition temperature using a Linear PlexZyme probe specific for Target 5.

In all of the exemplary strategies outlined in **Tables 1, 2 and 3**, and the discussion relating to these, the M-tec Probe included in the mix could be either an M-Tec-P Probe or an M-Tec-H Probe or an M-Tec E probe since all types exhibit similar properties with respect to their components and capacity to generate target dependent fluorescence at a first acquisition temperature and background only at a second acquisition temperature, provided the T_m OC1/OC2 is below the second acquisition temperature.

A strategy for combining an M-Tec-P Probe with a LOCS probe to analyse two targets at a single wavelength may be further understood by the illustration in **Figure 6**. **Figure 6** schematically illustrates an approach for multiplex analysis of two targets using the combination of one M-Tec-P Probe (A) and one LOCS probe (B) both of which are labelled with the same fluorophore (F) and quencher (Q). Reaction mixes contain an intact M-Tec-P Probe (Ai) composed of first and second oligonucleotide components, and an intact LOCS probe (Bi). In the presence of Target 1 (T1), PlexZyme 1 (P1) assembles and cleaves the sensor region in the intact M-Tec-P Probe to generate a cleaved M-Tec-P Probe (Ac). In the presence of Target 2 (T2), PlexZyme 2 (P2) assembles and cleaves the intact LOCS probe to generate a cleaved, Split LOCS Probe (Bc). **Figure 6** Panel (i) illustrates structures which can form at acquisition temperature 1 which is below the T_m OC1/OC2 of both intact and cleaved M-Tec-P Probes, and below the T_m of the stem of both intact and Split LOCS probe species. **Figure 6** Panel (ii) illustrates structures which can be formed at acquisition temperature 2 which is above the T_m OC1/OC2 of both intact and cleaved M-Tec-P probes, and above the T_m of the stem of the Split LOCS probe but below the T_m of the stem of the intact LOCS probe. At temperature 1 fluorescence above background would be generated in the presence of Target 1 but not in the absence of Target 1. At this temperature, the intact LOCS probe may be cleaved but no fluorescence above background would be generated from the resultant Split LOCS probe since the stem would remain hybridized and the fluorophore would remain quenched. Hence, an increase in detectable signal measured as fluorescence above background at temperature 1 would indicate the presence of Target 1 and background fluorescence would be the same regardless of the presence or absence of Target 2. At temperature 2, the first and second oligonucleotide components of both cleaved and intact M-Tec-P probe complexes would dissociate and contribute constantly to background fluorescence. At this temperature the stem of the Split LOCS probe, but not intact LOCS

probe, would dissociate resulting in an increase in fluorescence above background. Hence a detectable signal measured as an increase in fluorescence above background at temperature 2 would indicate the presence of Target 2 and background fluorescence would be the same regardless of the presence or absence of Target 1.

A strategy for combining an M-Tec-H Probe with a LOCS probe to analyze two targets at a single wavelength may be further understood by the illustration in **Figure 8**. **Figure 8** schematically illustrates an approach for multiplex analysis of two targets using the combination of one M-Tec-H Probe (A), comprising a first and a second oligonucleotide component, and one LOCS probe (B) both of which are labelled with the same fluorophore (F) and quencher (Q). Reaction mixes contain an intact M-Tec-H Probe (Ai) and an intact LOCS probe (Bi). In the presence of Target 1 (T1), the 5'-3' exonuclease activity of polymerase hydrolyses the sensor region in the intact M-Tec-H Probe to generate a modified M-Tec-H Probe (Ac). In the presence of Target 2 (T2), a PlexZyme (P) assembles and cleaves the intact LOCS probe to generate a cleaved, Split LOCS Probe (Bc). Panel (i) illustrates structures which can be formed at acquisition temperature 1 which is below the T_m OC1/OC2 of both the intact and cleaved M-Tec-H complexes, and below the T_m of the stem of both Split and Intact LOCS species. Panel (ii) illustrates structures which can be formed at acquisition temperature 2 which is above the T_m OC1/OC2 of both the intact and cleaved M-Tec-H complexes, and above the T_m of the stem of the Split LOCS but below the T_m of the stem of the Intact LOCS. At temperature 1 fluorescence above background would be generated in the presence of Target 1 but not in the absence of Target 1. At this temperature, the intact LOCS could be cleaved but no fluorescence above background would be generated from resultant Split LOCS probes since the stem would remain hybridized and the fluorophore would remain quenched. Hence an increase in fluorescence above background at temperature 1 would indicate the presence of Target 1 and background fluorescence would be the same regardless of the presence or absence of Target 2. At temperature 2, the first and second oligonucleotide components of both the intact and cleaved M-Tec-H complexes would dissociate and contribute constantly to background fluorescence. At this temperature, the stem of Split LOCS but not of intact LOCS probe, would dissociate resulting in an increase in fluorescence above background. Hence an increase in fluorescence above background at temperature 2 would indicate the presence of Target 2 and background fluorescence would be the same regardless of the presence or absence of Target 1.

Persons skilled in the art will recognize that M-Tec probes may detect the target directly or they may detect target amplicons following amplification of the target using

methods well known in the art. Further, persons skilled in the art will recognize that M-Tec probes may detect amplicons in real time or at the end of the reaction. By the way of example, a melt curve analysis could be used at the end of an amplification reaction containing M-Tec probes to determine the presence or absence of the specific target and/or its amplicon. Intact M-Tec probes remain quenched at a low temperature but produce fluorescence once the oligonucleotide components dissociate, for example OC1/OC2. At the melting temperature, where 50% of the M-Tec probes are dissociated, an observable peak would appear in the first derivative of the melting curve. Cleaved M-Tec probes may produce fluorescence below and above this melting temperature, and therefore would not have an observable peak at this temperature in the first derivative of the melting curve. Therefore, the presence of the melt peak at T_m OC1/OC2 would indicate the absence of the target, and the absence of the melt peak would indicate the presence of the target.

An M-Tec probe may be used in combination with one or more probes for a single channel multiplexing, which could be analysed with melt curve analysis. Multiple M-Tec probes with different T_m OC1/OC2 would produce differentiable melt peaks at each T_m OC1/OC2 in the absence of each target in the first derivative of the melting curve, which would not be observable in the presence of each target (see, e.g., **Figure 20**). Alternatively, an M-Tec probe may be used with other probes that produce signals in a temperature-dependent manner, as these probes produce melt peaks in the first derivative of the melting curve, which may only appear either in the presence or absence of the target. For example, a LOCS probe may produce a peak at a specific low temperature in the presence of the specific target, and at a specific higher temperature in the absence of the target. The analysis of the peaks produced by either intact or cleaved LOCS could be done simultaneously with the analysis of the peaks produced by M-Tec probes. Persons skilled in art will recognise that any possible combination of probe types could be used where more than two probes are used for analysis per fluorescent channel or wavelength, if each probe used in the reaction produce peaks that are differentiable in the melt curve analysis.

- *LOCS Oligonucleotides*

Exemplary LOCS oligonucleotides for use in the present invention are illustrated in **Figure 9**. The exemplary Intact LOCS oligonucleotide shown (**Figure 9A**, LHS) has a Loop region, a Stem region and a fluorophore (F)/quencher (Q) dye pair. Although exemplified with a fluorophore/quencher pair, the skilled addressee will recognise that

any other suitable detection moieties may be used for the same purpose. The Loop region contains a substrate region which is amenable to enzymatic cleavage or degradation in the presence of target or target amplicons. Cleavage or degradation of the Loop within an Intact LOCS generates the Split LOCS duplex (**Figure 9B**, RHS).

In some embodiments, the melting temperature ("T_m") of the Intact LOCS oligonucleotide is higher than the T_m of the Split LOCS structure.

Since intramolecular bonds are stronger than intermolecular bonds, the stem regions of the intact LOCS structures will generally melt at a higher temperature than the stems of the Split, cleaved or degraded LOCS oligonucleotide structures. For example, the Stem of intact LOCS A will melt at T_m A which is higher than T_m B which is the temperature at which Split LOCS stem melts (**Figure 9B**). The presence of fluorescence at a temperature which allows melting of Split LOCS but not Intact LOCS is indicative of the presence of target, or target amplicons. In the exemplary LOCS depicted in **Figure 10**, the sequence of the Loop region of a LOCS oligonucleotide may be, for example, a substrate for a PlexZyme or other catalytic nucleic acid/s.

An exemplary LOCS suitable for use in the invention may contain a Loop region comprising a substrate for a catalytic nucleic acid as illustrated in **Figure 10**. In these embodiments, LOCS oligonucleotides may comprise universal substrates which can be used to detect any target. The LOCS oligonucleotide contains a stem region, a fluorophore quencher/dye pair (alternative detection moieties as described herein may be employed) and an intervening Loop region which comprises a universal substrate for a catalytic nucleic acid such as an PlexZyme. The PlexZyme may detect a target directly or may be used to detect amplicons generated during target amplification. The PlexZyme forms when the target sensor arms of the partzymes each hybridise to a target, or to target amplicons, by complementary base pairing to form the active catalytic core of the PlexZyme. The Loop region of the LOCS oligonucleotide hybridises to the substrate binding arms of the PlexZyme by complementary base pairing and the substrate within the Loop is cleaved by the PlexZyme. This generates a Split LOCS structure which has a stem with a T_m B that is lower than the T_m A of the Intact LOCS. Measurement of a fluorescent signal at temperatures above T_m B but below T_m A is indicative of the presence of target in the reaction. Persons skilled in the art will recognize that the targets can be detected in real time or at the end of the reaction.

Other mechanisms for degradation of the loop region of the intact LOCS probes are known in the art. Alternative designs for LOCS probes have loop regions which can directly bind to the target. These types of LOCS probes can be degraded by exonuclease

activity of a polymerase or by a restriction endonuclease. It will be understood that the terms "LOCS" probe as used herein encompasses all known LOCS probes including those disclosed in the PCT patent publication numbers WO 2020/031156 A1 and WO2020206509A1 (the contents of each of these documents are incorporated herein by reference in their entirety).

- ***Further Exemplary Embodiments***

In certain embodiments, reporter oligonucleotides including M-Tec Probes of the present invention may be used to detect a target directly without being coupled to a target amplification protocol. In other exemplary embodiments reporter probes or substrates may be used to detect target amplicons generated by target amplification technologies including, but not limited to, PCR, RT-PCR, SDA, NEAR, HDA, RPA, LAMP, RCA, TMA, 3SR, LCR, RAM or NASBA. Cleavage or degradation of an M-Tec probe may occur in real time during target amplification or may be performed following amplification, at the end point of the reaction. The OC1 may be modified by target-dependent cleavage or degradation mediated by the enzymatic activity of a catalytic nucleic acid including, but not limited to a PlexZyme, an aptazyme, an apta-MNAzyme, a DNAzyme, a ribozyme, or by the enzymatic activity of a protein enzyme including an exonuclease or an endonuclease. By way of non-limiting example, the exonuclease activity may be an inherent catalytic activity of, for example, a polymerase. By way of non-limiting example, the endonuclease activity may be an inherent catalytic activity of, for example, a restriction enzyme including a Nicking endonuclease, a riboendonuclease or a duplex specific nuclease (DSN).

Reactions of the present invention may detect multiple targets simultaneously using a single M-Tec-P probe or probes in combination with other type(s) of reporter probes. As would be evident to persons skilled in the art, standard reporter probes can further be combined with additional M-Tec-P probes, for example, wherein each M-Tec-P probe may comprise a different universal substrate within its OC1.

The reaction mix may further comprise additional reporter probes or substrates combined with M-Tec probes labelled with different fluorophore and quencher pairs. By way of non-limiting example, a Reporter oligonucleotide 1 and M-Tec probe 2 may be labelled with fluorophore A, and Reporter oligonucleotide 3 and Intact M-Tec probe 4 may be labelled with fluorophore B. Target-dependent fluorescence associated Fluorophore A may be detected at multiple temperatures in Fluorescence channel A and target-dependent fluorescence associated Fluorophore B may be detected at multiple temperatures in fluorescence channel B on an instrument. The reaction mix may further

comprise at least one pair of reporter probes comprising a reporter probe or substrate and an M-Tec probe labelled with different fluorophores which emit fluorescence at a similar wavelength which can be monitored in a single fluorescent channel on an instrument.

In a further exemplary embodiment, a M-Tec Probe and a LOCS oligonucleotide may be combined wherein both contain the same fluorophore/quencher dye pair and the substrate regions are specific for a DNAzyme or a ribozyme, for example, a DNAzyme or ribozyme which can only be catalytically active in the presence of a specific metal ion. Specific DNAzymes and ribozymes are known in the art to require a metal cation cofactor to enable catalytic activity. For example, some DNAzymes and ribozymes can only be catalytically active in the presence of, for example, lead or mercury. Such metals may be present in, for example, an environmental sample. A reaction could include one M-Tec-P probe suitable for cleavage by a DNAzyme, which is, for example, mercury dependent, wherein the presence of mercury in a sample could result in cleavage of the substrate in the OC1 and generation of a fluorescent signal. The same reaction could also include a LOCS reporter which contains a loop comprising a substrate for a DNAzyme, which is, for example, lead dependent, wherein the presence of lead in a sample could result in cleavage of the LOCS and generation of a fluorescent signal at a temperature higher than the T_m of the split LOCS. An increase in fluorescence at a first temperature, which is below the T_m OC1/OC2 and below the T_m of the Split LOCS, would indicate the presence of mercury. An increase in fluorescence at a second temperature, which is above the T_m OC1/OC2 and the T_m of the Split LOCS but below the T_m of the Intact LOCS, would indicate the presence of lead. One skilled in the art would readily recognize that multiple probes cleavable in the presence of specific metal cofactors, could be combined in a single reaction and detected either in real time or at the end of the reaction.

Non-limiting examples of target nucleic acids (i.e. polynucleotides), which may be detected using M-Tec probes in combination with other well-known probes types could include DNA, methylated DNA, alkylated DNA, complementary DNA (cDNA), 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).

M-Tec-P probes could also be used to detect proteins or other molecules which can be recognized by aptamers incorporated into aptazymes or apta-MMAzymes. The catalytic activity of aptazymes or apta-MMAzyme may be inhibited in the absence of target molecules that are capable of binding to the aptamer domain of these nucleic acid

enzymes. Binding of a molecule, for example a protein, to the aptamer domain could activate the catalytic potential of an aptazyme or apta-MMAzyme which could facilitate cleavage of a first oligonucleotide component of an M-Tec-P probe.

Generation of Detectable Signals

The methods and compositions of the present invention utilise detection moieties to provide detectable signals. The nature of the detectable signal that the moieties are capable of producing will depend on the type of detection moiety and/or the conformation of the oligonucleotide to which it is associated.

Any suitable detection moiety can be utilised that is capable of providing a detectable signal upon the modification of an oligonucleotide to which it is associated. Non-limiting examples of suitable detection moieties include fluorophores for fluorescent signal generation, nanoparticles for colorimetric or SPR signal generation, reactive moieties (e.g. alkaline phosphatase or peroxidase enzymes) for chemiluminescent signal generation, electroactive species for electrochemical signal generation, and any combination thereof. By way of non-limiting example, suitable electroactive species include Methylene blue, Toluene Blue, Oracet Blue, ferrocene, Hoechst 33258, [Ru(phen)₃]²⁺ or Daunomycin and the most common electrode materials include gold, glassy carbon, pencil graphite or carbon ionic liquid. Methods for the detection and measurement of fluorescent, chemiluminescent, colorimetric, surface plasmon resonance (SPR) and electrochemical signals are well known to persons skilled in the art.

By way of non-limiting example, oligonucleotides of the present invention, including M-Tec probes, may have one or more fluorophores attached. The detectable signal inherently generated by the fluorophore may be quenched due to proximity to one or more quencher molecules. Fluorophores and quenchers are both referred to herein as detection moieties, but it will be understood that it is the fluorophore that emits a fluorescent signal when spatially separated from the quencher. For example, and without limitation, the fluorophore(s) may be attached to a single strand of a double-stranded stem portion (e.g. at the 5' or 3' terminus) of a Molecular Beacon or a LOCS, and the quencher(s) may be attached to an opposing strand of the double-stranded stem portion (e.g. at the 5' or 3' terminus). Alternatively, the quencher(s) may be attached to another entity (e.g. a surface or another oligonucleotide) to which the oligonucleotide is bound such that the detectable signal inherently generated by the fluorophore may be quenched. In the presence of a target, the oligonucleotide may undergo a modification that distances the fluorophore(s) from the quencher molecule(s) thus generating a detectable signal.

Additionally, or alternatively, the oligonucleotides (including OC1 or OC2) may be attached to gold nanoparticles (GNP) for colorimetric detection. When GNPs are aggregated in close proximity to each other they exhibit a purple colour (i.e. absorbance at a longer wavelength) and when GNPs are separated they exhibit a red colour (i.e. absorbance at a shorter wavelength) wherein, a measurable colour change from purple to red (e.g. M-Tec, LOCS, linear PlexZyme substrates, Catcher-Pitcher probes, TaqMan probes and restriction enzyme probes) or alternatively from red to purple (e.g. dual hybridisation probes) is indicative of the presence of a specific target in a sample.

Additionally, or alternatively, the oligonucleotides (including OC1 or OC2) and/or oligonucleotide components may be attached to a GNP and/or a gold surface for SPR detection of a target in a sample. When GNPs move into close proximity, or alternatively when they move away from a gold surface, they can generate a change in measurable SPR signal where a decrease in SPR signal using some approaches (e.g. M-Tec Probes, LOCS, linear PlexZyme substrates, TaqMan probes and restriction enzyme probes) can be indicative of the presence of a specific target in a sample or alternatively wherein an increase in SPR signal using other approaches (e.g. Catcher-Pitcher probes and dual hybridisation probes) can be indicative of the presence of a specific target in a sample.

Additionally, or alternatively, the oligonucleotide reporter and probes (including OC1 or OC2) and/or oligonucleotide components may be attached to electroactive species and/or on an electrode surface for electrochemical detection. When the oligonucleotides attached to electroactive species move into close proximity with, or alternatively when they move away from, an electrode surface they can generate a measurable change in oxidation or reduction current. In some embodiments (e.g. M-Tec Probes, LOCS, linear PlexZyme substrates, TaqMan probes and restriction enzyme probes), the resulting measurable signal arising from an electroactive species moving away from the electrode surface is indicative of the presence of a specific target in a sample. Alternatively, in other embodiments (e.g. Catcher-Pitcher probes and dual hybridisation probes), the resulting measurable signal arising from an electroactive species moving into close proximity to the electrode surface is indicative of the presence of a specific target in a sample.

Changes in colour, SPR signals, or electrochemical signals, in the presence or absence of targets may be manipulated by temperature in a similar manner to that described above for fluorescence detection.

In some embodiments, the compositions and methods of the present invention utilise M-Tec oligonucleotide components attached to a specific detection moiety in combination with another oligonucleotide probe that is attached to the same detection

moiety, or a similar detection moiety that generates a detectable signal capable of being detected simultaneously with signal generated by the detectable moiety of the M-Tec probe (e.g. using a single type of detector such as one fluorescence channel, or a specific mode of colorimetric, surface plasmon resonance (SPR), chemiluminescent, or electrochemical detection).

Analyses of Fluorescent Signals

Without limitation and by way of example only, detectable moieties used in accordance with the present invention include fluorescent signals generated by these detection moieties upon modification, cleavage or digestion of oligonucleotide probes to which they are attached, coupled, or otherwise associated, including dissociation or association of oligonucleotide components, or fragments of oligonucleotide components, of M-Tec complexes, can be analysed in any suitable manner to detect, differentiate, and/or quantify target molecules in accordance with the methods of the present invention.

By way of non-limiting example, measurements of fluorescent signal at a single temperature, or at multiple temperatures, may be obtained at various time points within a reaction suitable for detecting modification of M-Tec probe oligonucleotides. By way of non-limiting examples, these time points may comprise (i) a time point at, or near, the initiation of a reaction, and/or (ii) a single time point, or multiple time points, during the course of the reaction; and/or (iii) a time point at the conclusion or endpoint of the reaction.

In some embodiments, measurement of fluorescent signal may be obtained at two or more temperatures at each cycle during an amplification reaction, such as during PCR amplification. Analysis may be performed by comparing levels of fluorescence obtained at a first and/or second temperature and/or at a further temperature. By way of example, a first target may be detected using an M-Tec probe where fluorescent signals can be measured at multiple time points, or at multiple cycles, for example, at each cycle during PCR. In the same reaction, a second target may be detected using a LOCS probe where fluorescent signals can be measured at multiple time points, or at multiple cycles, for example, at each cycle during PCR. In such embodiments, quantitative data may be determined for both the first and second targets. By way of non-limiting example, the first M-Tec Probe may be cleaved by a first PlexZyme in the presence of a first target and monitored in real time, whereas a LOCS probe may be cleaved by a second PlexZyme in the presence of a second target and monitored in real time.

In several embodiments, measurement of fluorescent signal may be obtained at two temperatures in reactions which are tailored to measure two targets at the same wavelength.

In some embodiments a first target may be detected using an M-Tec probe where fluorescent signals can be measured at multiple time points, or at multiple cycles, for example, at each cycle during PCR. In the same reaction a second target may be detected using a LOCS by comparing pre-PCR and post-PCR fluorescence levels. In such embodiments, quantitative data may be determined for the first target, whilst qualitative data may be generated for the second target. By way of non-limiting example, the first M-Tec Probe may comprise a PlexZyme substrate cleaved by a first PlexZyme in the presence of a first target and monitored in real time at a single first temperature, whereas a LOCS probe may be cleaved by a second PlexZyme in the presence of a second target and monitored using endpoint detection analysis at a second temperature.

In some embodiments an increase in fluorescence at the first temperature is indicative of the presence of the first or second target and an increase in fluorescence at the second temperature is indicative of the presence of the second target. In other embodiments an increase in fluorescence at the first temperature is indicative of the presence of the first target and an increase in fluorescence at the second temperature is indicative of the presence of the second target. In other embodiments, measurement of fluorescent signal may be obtained at two or more temperatures at each cycle during PCR, and amplification curves may be plotted for each series of measurement obtained at each temperature. Threshold fluorescence values can be assigned to each amplification plot for each specific temperature and Ct or Cq values may be measured as the cycle number where the amplification plots cross the threshold values.

In embodiments wherein a first probe is an M-Tec probe for detection of target 1 and the second probe is a Linear PlexZyme Probe for detection of target 2; and wherein measurement of fluorescent signal is obtained at two temperatures at the same wavelength at each cycle during PCR, the total fluorescent signal from the M-Tec probe and linear PlexZyme substrate is measured at the lower temperature, which is below T_m OC1/OC2, and the detectable signal measured at a higher temperature, which is above T_m OC1/OC2, is solely from the linear PlexZyme substrate, the M-Tec probe signal can be calculated using the differential between the signal measured at the two temperatures, and the Cq value for target 1 can be determined thereafter; and the Cq value for target 2 can be determined from the signal measured at the higher temperature.

In other embodiments, measurement of fluorescent signal may be obtained at two or more temperatures at each cycle during PCR, and amplification curves may be plotted for each series of measurement obtained at each temperature. Threshold fluorescence values can be assigned to each amplification plot for each specific temperature and C_q values may be measured as the cycle number where the amplification plots cross the threshold values. In embodiments wherein a first probe for detection of a first target is an M-Tec probe, is combined with a second probe for detection of a second target, which may be a LOCS reporter; and wherein measurement of fluorescent signal is obtained at two temperatures at each cycle during PCR, the C_q measured using fluorescent signal from the first probe at the lower temperature, which is below the T_m OC1/OC2 and below the T_m of a Split LOCS, may allow direct quantification of the starting concentration of a first target; and the C_q measured using fluorescent signal from the LOCS reporter at the higher temperature, which is above the T_m OC1/OC2 and above T_m of the Split LOCS but below the T_m of the Intact LOCS, may allow direct quantification of the starting concentration of a second target.

By way of non-limiting example, baseline fluorescence signal can be obtained by measuring fluorescence at selected temperatures, for example a first and second temperature, at a time point which is either at, or near, the initiation of a reaction, for example pre-PCR. Prior to PCR and at a first temperature, a reporter probe, for example an M-Tec Probe, a linear PlexZyme substrate or a TaqMan probe or a Molecular Beacon, and the Intact LOCS would be quenched and not producing significant fluorescence signal, providing this temperature is below the T_m OC1/OC2 and the T_m of the stem of the Intact LOCS (and Molecular Beacon if present). Analysis may be performed by comparing levels of fluorescence obtained at the first and second temperature at a time point at the initiation of a reaction (e.g. pre-PCR) and levels of fluorescence obtained at the first and second temperatures at a time point, or time points, during and/or after the reaction (e.g. during PCR or post-PCR).

Exemplary applications of M-Tec probes when combined with other reporter/probes

- *Detection of targets during or following Target amplification*

M-Tec probes of the present invention may be used to determine the presence of amplified target nucleic acid sequences. No particular limitation exists in relation to amplification techniques to which the M-Tec probes may be applied. Amplicons generated by various reactions may be detected by M-Tec probes, provided the presence of target amplicons can promote the cleavage or degradation of an Intact M-Tec probe to

produce modified M-Tec probes. Non-limiting examples of methods useful in cleaving or degrading the sensor region of the first oligonucleotide components of M-Tec probes include cleavage by PlexZymes, DNazymes, aptazymes, Apta-MNazymes, ribozymes, restriction enzymes, endonucleases or degradation by exonucleases including but not limited to the exonuclease activity of a polymerase.

In general, nucleic acid amplification techniques utilise enzymes (e.g. polymerases) to generate copies of a target nucleic acid that is bound specifically by one or more oligonucleotide primers. Non-limiting examples of amplification techniques in which M-Tec probes may be used include one or more of the polymerase chain reaction (PCR), the reverse transcription polymerase chain reaction (RT-PCR), strand displacement amplification (SDA), helicase dependent amplification (HDA), Recombinase Polymerase Amplification (RPA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), transcription-mediated amplification (TMA), self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), Ligase Chain Reaction (LCR) or Ramification Amplification Method (RAM).

The skilled addressee will readily understand that the applications of M-Tec probes described above are provided for the purpose of non-limiting exemplification only. The M-Tec probes disclosed may be used in any primer-based nucleic acid amplification technique and the invention is not so limited to those embodiments specifically described.

- *Detection of amplicons generated using M-Tec probes*

As discussed above, M-Tec probes of the present invention may be utilised in any polynucleotide amplification technique, non-limiting examples of which include the PCR, RT-PCR, SDA, HDA, RPA, LAMP, RCA, TMA, RAM, LCR, 3SR, or NASBA.

Amplicons generated by these techniques may be detected utilizing M-Tec probes which may be cleaved or degraded using any suitable method known in the art. Non-limiting examples include the use of catalytic nucleic acids, exonucleases, endonucleases and the like.

A PlexZyme may be utilised to generate cleaved or modified M-Tec probes by detecting amplicons generated through methods such as PCR, RT-PCR, SDA, HDA, RPA, TMA, LAMP, RCA, LCR, RAM, 3SR, and NASBA. The PlexZyme may comprise one or more partzyme(s). PlexZymes are multi-component nucleic acid enzymes which are assembled and are only catalytically active in the presence of an assembly facilitator which may be, for example, a target to be detected such as an amplicon generated from a polynucleotide sequence using primers. PlexZymes are composed of multiple part-

enzymes, or partzymes, which self-assemble in the presence of one or more assembly facilitators and form active PlexZymes which catalytically modify substrates. The substrate and assembly facilitators (target) are separate nucleic acid molecules. The partzymes have multiple domains including (i) sensor arms which bind to the assembly facilitator (such as a target nucleic acid); (ii) substrate arms which bind the substrate, and (iii) partial catalytic core sequences which, upon assembly, combine to provide a complete catalytic core. PlexZymes can be designed to recognize a broad range of assembly facilitators including, for example, different target nucleic acid sequences. In response to the presence of the assembly facilitator, PlexZymes modify their substrates. This substrate modification can be linked to signal generation and thus PlexZymes can generate an enzymatically amplified output signal. The assembly facilitator may be a target nucleic acid present in a biological or environmental sample (e.g. an amplicon generated from a polynucleotide target using primers). In such cases, the detection of the modification of the substrate by the PlexZyme activity is indicative of the presence of the target. Several PlexZymes capable of cleaving nucleic acid substrates are known in the art. PlexZymes and modified forms thereof are known in the art and disclosed in 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).

Use of M-Tec probes as internal calibrator for machine-to-machine variation or well-to-well variation

An M-Tec probe could be used as an internal calibrator, since fluorescent signal could be generated by heating the reaction to above the T_m OC1/OC2 of the probe, and the signal could be quenched by lowering the reaction temperature below the T_m OC1/OC2, independent of the presence of the target in the reaction. Therefore, if the measurements would be taken prior to target amplification where the M-Tec probe would be intact, the difference between the measured values could function as passive reference signal, which could be used for signal normalization to account for the well-to-well variations.

A calibrator method that uses an M-Tec probe would have several advantages over other approaches including that it would not require the use of additional reagents to be added to the reaction nor would it require the use of data obtained from other wells. This method would function to calibrate and correct for well-to-well variations that may be

present. Furthermore, the calibration would be processed using the data acquired in the same channel and therefore would not be affected by any channel-to-channel variations that may be present within the instrument. Where multiple channels are utilized for a multiplex reaction, each channel could be independently calibrated against the M-Tec probe signal in each channel. This would be favorable to a scenario where the signals are calibrated against signals in a different channel, such as conventional passive reference dye or signals from the internal control or endogenous control, as the calibration is adversely affected if the ratio of the expected signal intensity between the channels differs significantly between the instruments, causing channel-to-channel variations.

- *Diagnostic and related applications*

Methods using M-Tec Probes optionally in combination with LOCS oligonucleotides and/or other well-known report probes may be used for diagnostic and/or prognostic purposes in accordance with the methods described herein. The diagnostic and/or prognostic methods may be performed *ex vivo* or *in vitro*. However, the methods of the present invention need not necessarily be used for diagnostic and/or prognostic purposes, and hence applications that are not diagnostic or prognostic are also contemplated. The methods and probes described herein may be used in the design and application of logic gates, biosensors and/or nanosensors (see, e.g., Yin *et al.*, (2020) *VIEW* 2(2); Xi *et al.*, (2022) *RSC Adv.* 12, 27421-27430).

In some embodiments, the methods described herein may be used to diagnose infection in a subject. For example, the methods may be used to diagnose infection by bacteria, viruses, fungi/yeast, protists and/or nematodes in the subject. In one embodiment, the virus may be an enterovirus. The subject may be a bovine, equine, ovine, primate, avian or rodent species. For example, the subject may be a mammal, such as a human, dog, cat, horse, sheep, goat, or cow. The subject may be afflicted with a disease arising from the infection. For example, the subject may have meningitis arising from an enterovirus infection. Accordingly, methods of the present invention may in certain embodiments be used to diagnose meningitis.

In some examples, the present disclosure provides use of a multi-component temperature-controlled probe as described herein in the preparation of a composition or kit for use in a method of detecting a target, such as a nucleic acid target. In some examples, the present disclosure provides use of a multi-component temperature-controlled probe as described herein in the preparation of a composition or kit for use in a method of diagnosing an infection. In some examples, the present disclosure provides use

of a multi-component temperature-controlled probe for detecting a target nucleic acid in the preparation of a composition or kit for diagnosing a disease.

The methods of the present invention may be performed on a sample. The sample may be derived from any source. For example, the sample may be obtained from an environmental source, an industrial source, or by chemical synthesis.

It will be understood that a “sample” as contemplated herein includes a sample that is modified from its original state, for example, by purification, dilution or the addition of any other component or components.

The methods of the present invention including, but not limited to diagnostic and/or prognostic methods, may be performed on a biological sample. The biological sample may be taken from a subject. Stored biological samples may also be used. Non-limiting examples of suitable biological samples include whole blood or a component thereof (e.g. blood cells, plasma, serum), urine, cervico-vaginal mucus, stool, saliva, lymph, bile fluid, sputum, tears, cerebrospinal fluid, bronchioalveolar lavage fluid, synovial fluid, semen, ascitic tumour fluid, breast milk and pus.

Kits

The present invention provides kits comprising one or more agents for performing methods of the present invention. Typically, kits for carrying out the methods of the present invention contain all the necessary reagents to carry out the method.

In some embodiments the kits may comprise oligonucleotide components capable of forming one or more PlexZymes in the presence of an appropriate assembly facilitator(s) (e.g. an amplicon as described herein) and/or one or more M-Tec probes. For example, the kit may comprise a first container comprising at least a first and a second oligonucleotide component of an M-Tec probe and a second container comprising a third and a fourth oligonucleotide component comprising partzymes, wherein self-assembly of the third and a fourth partzymes, into a PlexZyme requires association of a first assembly facilitator (e.g. an amplicon) present in a test sample. Optionally, for example, the kit may comprise at least a fifth and sixth oligonucleotide component comprising a third and fourth partzyme, and a second container comprising a LOCS substrate, wherein self-assembly of the third and fourth partzymes, and the LOCS substrate, into an PlexZyme LOCS complex requires association of a second assembly facilitator (e.g. an amplicon) present in a test sample. Accordingly, in such an embodiment, the first and second partzymes, and an oligonucleotide component for a substrate within the Loop region, may be applied to the test sample in order to determine the presence of one or more target

amplicons. In general, the kits comprise at least a first and second oligonucleotide component for an M-Tec probe. provided herein. In some examples, the kit comprises a first oligonucleotide component and a second oligonucleotide component of an M-Tec probe. The kit may comprise a first oligonucleotide component, a second oligonucleotide component and a third oligonucleotide component of an M-Tec probe. In some examples, the kit comprises a first container comprising a first component oligonucleotide, a second component oligonucleotide and optionally a third oligonucleotide of an M-Tec probe (e.g., an M-Tec-P probe), and a second container comprising partzyme oligonucleotides capable of self-assembly into a PlexZyme in the presence of a first assembly facilitator (e.g., an amplicon). In some examples, the kit comprises a first container comprising a first oligonucleotide component, a second oligonucleotide component and optionally a third oligonucleotide component of an M-Tec probe (e.g., an M-Tec-H probe), and a second container comprising an exonuclease or a polymerase having exonuclease activity. In some examples, the kit comprises a first container comprising a first oligonucleotide component, a second oligonucleotide component and optionally a third oligonucleotide component of an M-Tec probe (e.g., an M-Tec-E probe), and a second container comprising an endonuclease, such as a restriction enzyme or a nicking endonuclease.

Typically, the kits of the present invention will also comprise other reagents, wash reagents, enzymes and/or other reagents as required in the performance of the methods of the invention such as PCR or other nucleic acid amplification techniques.

The kits may be fragmented kits or combined kits as defined herein.

Fragmented kits comprise reagents that are housed in separate containers, and may include 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 the samples and reagents, and the addition of agents or solutions of each container from one compartment to another in a quantitative fashion.

Such kits may also include a container which will accept the test sample, a container which contains the reagents used in the assay, containers which contain wash reagents, and containers which contain a detection reagent.

Combined kits comprise all of the components of a reaction assay in a single container (e.g. in a single box housing each of the desired components).

A kit of the present invention may also include instructions for using the kit components to conduct the appropriate methods. Kits and methods of the invention may

be used in conjunction with automated analysis equipment and systems, for example, including but not limited to, real time PCR machines.

For application to amplification, detection, identification or quantitation of different targets, a single kit of the invention may be applicable, or alternatively different kits, for example containing reagents specific for each target, may be required. Methods and kits of the present invention find application in any circumstance in which it is desirable to detect, identify or quantitate any entity.

It will be appreciated by persons of ordinary skill in the art that numerous variations and/or modifications can be made to the present invention as disclosed in the specific embodiments without departing from the spirit or scope of the present invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Examples

The present invention will now be further described in greater detail by reference to the following specific examples, which should not be construed as in any way limiting the scope of the invention.

Example 1: Detection of a target using an M-Tec-P probe which generates signal at one temperature only.

The following example demonstrates how an M-Tec-P probe can be used to detect the presence a target (*Chlamydia trachomatis*; CT) by monitoring increases in the fluorescence signal at a first temperature (52°C). Fluorescence is generated in the presence, but not in the absence of, the target at the first temperature, while showing no change in signal at a second higher temperature (76°C) regardless of the presence or absence of the target.

Oligonucleotides

The oligonucleotides for amplification and detection of Target 1 (CT). specific to this experiment include: Forward Primer 1 (SEQ ID: 1) Reverse Primer 1 (SEQ ID: 2), Partzyme A1 (SEQ ID: 3), Partzyme B1 (SEQ ID: 4), OC1/1-Q1 (SEQ ID: 5), OC2/1-SUN (SEQ ID: 6).

Reaction conditions

Real-time qPCR detection of the target sequence was performed in a total reaction volume of 20 µL using a Bio-Rad® CFX96 thermocycler. The cycling parameters were 95°C for 30 seconds, 50 cycles of 95°C for 5 seconds, 52°C for 40 seconds, 61°C for

10 seconds, and 76°C for 3 seconds. Fluorescence data was acquired in the HEX Channel at both the 52°C and 76°C at each PCR cycle. All reactions were run in duplicates and contained 40 nM of Forward Primer 1, 200 nM of each of Reverse Primer 1, Partzyme A1, Partzyme B1, OC1/1-Q1 and OC2/1-SUN, 1× NH₄ buffer (Meridian Bioscience), 8 mM MgCl₂ (Sigma-Aldrich), 800 μM dNTP mix (Meridian Bioscience) and 2 U AptaTaq exo DNA polymerase (Roche CustomBiotech).

The reactions either contained no target (NF H₂O), or 10000, 400 or 10 copies of synthetic double stranded DNA fragments (IDT), which is homologous to the target gene (CT). All reactions except for the no target control (NF H₂O) contained a background of 35 ng of human genomic DNA (Promega).

Results

During PCR amplification, fluorescence was measured at two temperatures in real-time. The presence of CT target was detected and monitored by the increase in fluorescence acquired at 52°C. No change in fluorescence signal was observed at 76°C, regardless of the presence or absence of the target. The signal observed at 52°C was generated by cleavage of substrate sequence within the OC1 by PlexZymes assembled in the presence of target CT template. Cleavage resulted in separation of the fluorophore and quencher moieties in the presence, but not in the absence, of target as illustrated schematically in **Figure 3 (i) and (ii)** respectively. The first temperature of 52°C is below the T_m OC1/OC2 and hence OC2 remains hybridized to the capture region of either a first fragment of OC1 when target is present (**Figure 3 (i)**), or an intact uncleaved OC1 in the absence of target (**Figure 3 (ii)**). No change in signal was observed at 76°C because all reactions give the same constant baseline fluorescence regardless of whether target is present or absent as illustrated schematically in **Figure 3 (iii) and (iv)** respectively. Background fluorescence at the high temperature is contributed by separation of OC1 and OC2 because 76°C is above the T_m OC1/OC2.

Experimental results in **Figure 11** shows amplification curves where fluorescence was acquired at 52°C (LHS) and 76°C (RHS) for reactions containing an M-Tec-P probe specific to CT and various numbers of copies of CT target or no target. At 52°C there was an increase in fluorescence in the presence of CT (A, B, C; black solid line) whereas there was no increase in fluorescence in reactions containing no target (D; grey solid line). The threshold cycle (C_t) value has a linear relationship with the logarithmic of the copy number of the target, as the average value of C_t for 10000 (A), 400 (B) or 10 (C) copies were 22.51, 27.13 and 32.37, respectively. Data acquired at 76°C showed no increase in

fluorescent signal over 50 PCR cycles in the same reactions regardless of the presence or absence of the target.

This example demonstrates that the M-Tec-P Probes allow target-specific fluorescence signal to be detected at a designated temperature in real-time. Further, it showed that the M-Tec-P Probe can be completely “switched off” at a higher temperature, demonstrating the capacity of this probe type to control signal generation at various temperatures. The M-Tec Probe showed high sensitivity, detecting as low as 10 copies while maintaining a robust signal across the linear dynamic range.

Example 2: Comparative performance of an M-Tec probe and a Molecular Beacon using Taq polymerase with and without exonuclease activity.

The following example compares the performance of an M-Tec-P probe and a Molecular Beacon, both of which are designed to detect a target (*Trichomonas vaginalis*; TV) by monitoring fluorescence at a first temperature (52°C) whilst showing no increase in signal at a second higher temperature (76°C), regardless of the presence or absence of the target. Probe performance is compared in reactions where different polymerases mediate PCR. The two polymerases used in this example were AptaTaq DNA polymerase, which has 5′-3′ exonuclease activity, and AptaTaq exo DNA polymerase, which does not have 5′-3′ exonuclease activity.

Oligonucleotides

The oligonucleotides in this experiment which are specific for amplification and detection of *Trichomonas vaginalis* (TV) include: Forward Primer 2 (SEQ ID: 7), Reverse Primer 2 (SEQ ID: 8), Partzyme A2 (SEQ ID: 9), Partzyme B2 (SEQ ID: 10), OC1/2-Q1 (SEQ ID: 11), OC2/2-FAM (SEQ ID: 12), Molecular Beacon 1 (SEQ ID: 13).

Reaction Conditions

Real-time amplification and detection were performed in a total reaction volume of 20 µL using a Bio-Rad® CFX96 thermocycler. The cycling parameters and fluorescent data acquisition (DA) points were: 1 cycle of 95°C for 30 seconds, 50 cycles of 95°C for 5 seconds, 52°C for 40 seconds, 61°C for 10 seconds and 76°C for 3 seconds. Fluorescence data was acquired in the FAM Channel at both 52°C and 76°C at each PCR cycle. All reactions were run in duplicate and contained 40 nM Forward Primer 2, 200 nM Reverse Primer 2, 1× NH₄ buffer (Meridian Bioscience), 8 mM MgCl₂ (Sigma-Aldrich), 800 µM dNTP mix (Meridian Bioscience) and either 2 units of AptaTaq DNA

polymerase (Roche CustomBiotech) or 2 units of AptaTaq exo DNA polymerase (Roche CustomBiotech). Molecular Beacon reactions contained 200 nM Molecular Beacon 1, whilst M-Tec-P reactions contained 200 nM each of Partzyme A2, Partzyme B2, OC1/2-Q1 and OC2/2-FAM.

The reactions either contained no target (NF H₂O), or 10000 or 100 copies of synthetic double stranded DNA fragments (IDT), which is homologous to the target gene (TV). All reactions except for the no target control (NF H₂O) contained a background of 35 ng of human genomic DNA (Promega). Ct values were determined using single threshold method with the thresholds for the amplification curves set at 75 RFU for 52°C and 30 RFU for 76°C on Bio-Rad® CFX Manager 3.1 software.

Results

During PCR amplification, fluorescence was acquired during each cycle in real-time at two temperatures, 52°C and 76°C. Amplification plots for data acquired at 52°C from reactions containing AptaTaq exo DNA polymerase (**Figure 12A**) showed an increase in fluorescence above background in the presence TV target ((i) 10000 and (ii) 100 copies), but not in the absence of target (iii), using both the M-Tec-P Probe (**Figure 12A MT-52**) and the Molecular Beacon (**Figure 12A MB-52**). Data acquired at 76°C showed no increase in fluorescence signal above background for either probe regardless of the presence or absence of the target (**Figure 12A; MT-76 and MB-76**) following stabilisation of baseline background fluorescence in the first few cycles.

When amplification was facilitated by AptaTaq exo DNA polymerase, the M-Tec-P probe reactions had earlier Ct values than the Molecular Beacon in the presence of target as shown in **Table 4** and in **Figure 12A MT/MB-52**. The ΔC_t between the M-Tec-P probe and Molecular Beacon were 2.90 and 2.59 for 10000 and 100 copies respectively. The M-Tec-P probe amplification curves were associated with a more rapid increase in fluorescence than the Molecular Beacon, had a larger dynamic range, and plateaued completely.

Table 4: *Ct values generated during PCR when target was detected using an M-Tec-P probe and Molecular Beacon using two polymerases. N/A equals Not Applicable (no Ct observed).*

Enzyme	Probe Type	Copies of TV per reaction	Average Ct at 52°C	Average Ct at 76°C
AptaTaq exo DNA polymerase	M-Tec-P probe	10000	24.14	N/A
		100	29.20	N/A
		No target	N/A	N/A
	Molecular Beacon	10000	27.04	N/A
		100	31.79	N/A
		No target	N/A	N/A
AptaTaq DNA polymerase	M-Tec-P probe	10000	24.27	N/A
		100	28.94	N/A
		No target	N/A	N/A
	Molecular Beacon	10000	25.69	24.71
		100	30.40	29.71
		No target	45.22	45.71

Amplification plots for data acquired at 52°C from reactions containing AptaTaq DNA polymerase (**Figure 12B**) showed an increase in fluorescence above background in the presence TV target (10000 (i) and 100 (ii) copies) using both the M-Tec-P Probe (**Figure 12B MT-52**) and the Molecular Beacon (**Figure 12B MB-52**). Further, as seen with the other polymerase, the M-Tec-P probe reactions produced earlier Ct values than Molecular Beacon in presence of target as shown in **Table 4** and in **Figure 12B (MT/MB-52)**. The ΔC_t between the M-Tec-P probe and Molecular Beacon were 1.42 and 1.46 for 10000 and 100 copies respectively. The M-Tec-P probe amplification curves were steeper than those from the Molecular Beacon and they plateaued completely. At 52°C, in the absence of target, no amplification was observed with the M-Tec-P probe; however low-level fluorescence, generating a late Ct value, was seen in reactions containing the Molecular Beacon indicating some non-specific signal. Data acquired at 76°C showed no increase in fluorescence signal above background for the M-Tec-P Probe regardless of the presence or absence of the target (**Figure 12B; MT-76**) following stabilisation of baseline background fluorescence in the first few cycles. In contrast, data acquired at 76°C showed an increase in fluorescence signal above background for the

Molecular Beacon both in the presence and absence of the target (**Figure 12B; MT-76**), indicating some non-specific signal. The Ct values for target specific signal were similar for data acquired at 52°C and 76°C. This is undesirable and indicates that fluorescence, which can be attributed to hydrolysis of Molecular Beacons by the 5'-3' exonuclease activity of the polymerase when the Molecular Beacon is bound to the target, is occurring. As such, the conditions whereby one can completely switch off Molecular Beacons at elevated temperatures is restricted and will likely depend on several factors including the properties of the polymerase selected and the melting temperatures of Molecular Beacon loop/Target hybrids. While useful under specific condition, Molecular Beacons have considerably more constraints than M-Tec-P probes for use in these protocols and hence are inferior for this purpose. Further, data in this experiment indicated the M-Tec-P probe may be more efficient at detection as evidenced by earlier Ct values and steeper amplification curves. Finally, data in this experiment also indicated the M-Tec-P probe may be more specific than Molecular Beacons.

Example 3: Real-time detection and quantification of two targets at a single wavelength using a combination of an M-Tec Probe and a LOCS probe.

The following example demonstrates how the combination of one M-Tec Probe and one LOCS reporter can allow simultaneous detection and quantification of two targets in a single fluorescent channel by acquiring fluorescence at two temperatures in real-time during PCR. Both the M-Tec Probe and the LOCS probe are labelled with the same fluorophore and quencher moieties for simultaneous detection in the same fluorescence channel. Each probe is designed to produce detectable signal at one temperature only, without any crosstalk to the other temperature.

In this example, the same pair of probes, one M-Tec-P probe and one LOCS Probe, were combined and used in two separate reactions, each to simultaneously detect two targets at two temperatures at the same wavelength during PCR.

The first reaction (Mix 1) included (i) an M-Tec-P Probe which was designed to be cleaved by a first PlexZyme in the presence of a first target *Trichomonas vaginalis* (TV) and to generate fluorescence above the baseline at a first temperature (52°C) only, and (ii) a LOCS reporter which was designed to be cleaved by a second PlexZyme in the presence of a second target the human TFRC gene in human genomic DNA (gDNA) and to generate fluorescence above the baseline at a second temperature (76°C) only.

The second reaction (Mix 2) included (i) an M-Tec-P Probe which was designed to be cleaved by a first PlexZyme in the presence of a first target *Neisseria gonorrhoeae* (NG) and to generate fluorescence above the baseline at a first temperature (52°C) only, and (ii) a LOCS reporter which was designed to be cleaved by a second PlexZyme in the presence of a second target *Chlamydia trachomatis* (CT) and to generate fluorescence above the baseline at a second temperature (76°C) only.

Oligonucleotides

The oligonucleotides specific to this experiment include: Forward Primer 1 (SEQ ID: 1), Reverse Primer 1 (SEQ ID: 2), Forward Primer 2 (SEQ ID: 7), Reverse Primer 2 (SEQ ID: 8), Partzyme A2 (SEQ ID: 9), Partzyme B2 (SEQ ID: 10), OC1/2-Q1 (SEQ ID: 11), OC2/2-FAM (SEQ ID: 12), Forward Primer 3 (SEQ ID: 14), Reverse Primer 3 (SEQ ID: 15), Partzyme A3 (SEQ ID: 16), Partzyme B3 (SEQ ID: 17), Forward Primer 4 (SEQ ID: 18), Reverse Primer 4 (SEQ ID: 19), Partzyme A4 (SEQ ID: 20), Partzyme B4 (SEQ ID: 21), Partzyme A5 (SEQ ID: 22), Partzyme B5 (SEQ ID: 23) and LOCS-1-FAM/Q1 (SEQ ID: 24).

Reaction conditions

Real-time detection of the target sequence was performed in a total reaction volume of 20 µL using a Bio-Rad® CFX96 thermocycler. The cycling parameters were 95°C for 30 seconds, 50 cycles of 95°C for 5 seconds, 52°C for 40 seconds, 61°C for 10 seconds, and 76°C for 3 seconds (data collected at both the 52°C and 76°C steps). All reactions were run in triplicates and contained 200 nM of OC1/2-Q1, OC2/2-FAM and LOCS-1-FAM/Q1, 1× NH₄ buffer (Meridian Bioscience), 8 mM MgCl₂ (Sigma-Aldrich), 800 µM dNTP mix (Meridian Bioscience) and 2 U AptaTaq DNA polymerase (Roche CustomBiotech). The reactions in Mix 1 contained 40 nM of Forward Primers 2 and 3, and 200 nM each of Reverse Primers 2 and 3, Partzymes A2, B2, A3 and B3. The reactions in Mix 2 contained 40 nM of Forward Primers 1 and 4, and 200 nM each of Reverse Primers 1 and 4, Partzymes A4, B4, A5 and B5.

The reactions in Mix 1 contained either 10000, 800 or 40 copies of synthetic double stranded DNA fragments (IDT) homologous to the target gene for TV only; or 10000, 800 or 40 copies of the TFRC gene present in human genomic DNA only (gDNA; Promega); or 10000, 800 or 40 copies of both TV and the TFRC gene in gDNA; or no target (NF H₂O only). The reactions in Mix 2 either contained either 10000, 800 or 40 copies of synthetic double-stranded DNA fragments (IDT) homologous to the target gene for NG only, or CT only, or both NG and CT; or no target (NF H₂O only).

Results

During PCR, fluorescence was measured at two temperatures in real-time (**Figure 13; Table 5**). In reaction Mix 1, the presence of a first target TV was detected at 52°C only (**Figure 13A**), and the presence of a second target, the TFRC gene, was detected at 76°C only (**Figure 13B**). In reaction Mix 2, the presence of a first target NG was detected at 52°C only (**Figure 13C**), and the presence of a second target CT was detected at 76°C only (**Figure 13D**).

Table 5: Threshold cycle Values (Ct values) for reactions containing one or two targets with acquisition at 52°C and 76°C.

		52°C			76°C		
Target 1 = T1 Target 2 = T2	Copy Number	T1	T1 + T2	T2	T1	T1 + T2	T 2
Mix 1 T1 = TV T2 = TFRC	10000	22.23	22.38	0	0	23.82	23.68
	800	26.75	26.54	0	0	27.31	27.75
	40	30.57	30.93	0	0	31.65	31.52
	0	0	0	0	0	0	0
Mix 2 T1 = NG T2 = CT	10000	23.15	23.04	0	0	24.01	24.21
	800	26.83	26.83	0	0	28.21	28.32
	40	31.31	30.95	0	0	32.25	32.03
	0	0	0	0	0	0	0

Figure 13A shows real-time amplification curves acquired at 52°C in the FAM channel of the Bio-Rad® CFX96 thermocycler with Mix 1. There were increases in fluorescence in reactions that contained TV only (black solid line), and those containing both TV and TFRC/gDNA (grey dashed line), but not in reactions lacking any target (grey solid line) or containing gDNA only (black dashed line). **Figures 13B** shows the amplification curves acquired at 76°C in the same channel. At this temperature there was increases in fluorescence in the reactions that contained TFRC/gDNA only (black dashed line), and those containing both TV and TFRC/gDNA (grey dashed line), but not in reactions lacking any target (grey solid line) or containing TV only (black solid line). The Ct values has a linear relationship with the logarithmic of the copy number of the target at both temperatures.

The amplification plots show similar Ct value at 52°C for TV only and for TV and TFRC/gDNA combined; and at 76°C for TFRC/gDNA only, and for TV and

TFRC/gDNA combined (**Figure 13A and B; Table 5**). This confirms that the fluorescence observed at 52°C is generated by the M-Tec-P probe only in the presence of TV and that the fluorescence observed at 76°C is generated by the LOCS probe only in the presence of the TFRC gene present in gDNA.

Similarly **Figures 13C** shows real-time amplification curves acquired at 52°C in the FAM channel obtained using with Mix 2. There were increases in fluorescence in reactions that contained NG only (black solid line), and those containing both NG and CT (grey dashed line), but not in reactions lacking target (grey solid line) or containing CT only (black dashed line). **Figures 13D** shows the amplification curves acquired at 76°C in the same channel. At this temperature there was increases in fluorescence in the reactions that contained CT only (black dashed line), and those containing both NG and CT (grey dashed line), but not in reactions lacking targets (grey solid line) or containing NG only (black solid line). Similar to results from the first mix, the Ct values have a linear relationship with the logarithmic of the copy number of the target at both temperatures.

The amplification plots show similar Ct value at 52°C for NG only and for NG and CT combined; and at 76°C for CT only, and for CT and NG combined (**Figure 13C and D; Table 5**). This confirms that the fluorescence observed at 52°C is generated by the M-Tec-P probe only in the presence of NG and that the fluorescence observed at 76°C is generated by the LOCS probe only in the presence of CT.

This example demonstrates that combining M-Tec-P Probe reporter and LOCS reporter in a single reaction allows target-specific fluorescence signal to be simultaneously detected at each designated temperature in real-time. This example shows that the co-amplification of another target to be detected at the same wavelength did not affect the specific signal at both temperatures. This indicates that there was no detectable crosstalk signal between temperatures when two targets are being detected and differentiated in the same fluorescent channel. For both reaction Mix 1 and Mix 2, the Ct values for each of the first targets were the same as those for each of the first target combined with an equal number of copies of the second target at the first temperature, and the Ct values for each of the second targets were the same as those for each of the second target combined with an equal number of copies of the first target at the second temperature. This indicates that each of the two probe types specifically detected only one target at one temperature. This example also shows that the same set of universal M-Tec Probe and LOCS reporter can be used for the detection of different target gene pairs. Both

probes are independent of target sequence and can therefore be easily implemented into any assay for the detection of any desired target gene or transcript.

Some of the other methods known in the art, which allow detection of multiple targets at a single wavelength, generate fluorescence at one of two temperatures which is associated with multiple probe types. These methods involve mathematical manipulation to extract the signal arising from the specific assay and have the crosstalk signals removed post-reaction. Similarly, the ChromaCode strategy requires manipulation of the data from a single temperature to extract the desired data for individual targets (see, e.g., International Patent Publication No. WO/2017/173035). In contrast the strategy demonstrated in this example eliminates the requirement for mathematical manipulation of the data. Further, the use of M-Tec-P probes combined with LOCS probes provides an approach whereby both probes are universal for any target. This has advantages as they can be synthesised in bulk, thus reducing costs, and they can be held in stock so that they are always readily available for rapid development of new assays.

Example 4: An M-Tec-H that generates a target-specific signal arising from the 5'-3' exonuclease activity which is detectable at a first temperature but not at a second higher temperature

The following example demonstrates how an M-Tec-H probe can be used to detect the presence of a target (*Mycoplasma genitalium*; MG) by monitoring increases in the fluorescence signal at a first temperature (52°C). Fluorescence can be generated in the presence, but not in the absence of, the target at the first temperature, while showing no change in signal at a second higher temperature (76°C), regardless of the presence or absence of the target.

Oligonucleotides

The oligonucleotides specific to this experiment include: Forward Primer 5 (SEQ ID: 25), Reverse Primer 5 (SEQ ID: 26), OC1/3-Q1 (SEQ ID: 27) and OC2/3-SUN (SEQ ID: 28) for amplification and detection of the MG target, and Aptamer 1 (SEQ ID: 29); the sequences of which are listed in the Sequences Listing.

Reaction conditions

Real-time detection of the target sequence was performed in a total reaction volume of 20 µL using a Bio-Rad® CFX96 thermocycler. The cycling parameters were 95°C for 30 seconds, 50 cycles of 95°C for 5 seconds, 52°C for 40 seconds, 61°C for 10 seconds, and 76°C for 3 seconds (with fluorescent data acquisition at both the 52°C

and 76°C steps). All reactions were run in duplicate and contained 200 nM of Forward Primer 4, Reverse Primer 4, OC1/3-Q1 and OC2/3-SUN, 50 nM Aptamer 1, 1× SensiFAST 16 Probe No-ROX Kit (Meridian Bioscience).

The reactions contained either 10000 or 40 copies of synthetic double-stranded DNA fragments (IDT) homologous to the target gene for MG present in a background of 35 ng of human genomic DNA (Promega), while No Template control reaction contained no target and NF H₂O only.

Results

During PCR amplification, fluorescence was measured at two temperatures in real-time in the HEX channel (**Figure 14**). At 52°C, the presence of the MG target was detected as shown in the amplification curves for 10000 copies (i) and 40 copies (ii) in **Figure 14A**. No increase in fluorescence was observed in the absence of template (iii). The average threshold cycle (Ct) value for 10000 and 40 copies were 25.58 and 32.96, respectively. Following stabilisation of background fluorescence in the first few cycles, no change in signal was observed at 76°C regardless of the presence or absence of the target MG (**Figure 14B**).

The first temperature, 52°C, was chosen to be below the temperature at which the two oligonucleotide components OC1 and OC2 of the M-Tec-H probe can hybridize; specifically, it was less than the T_m OC1/OC2. At this temperature, intact OC1 which is labelled with a quencher could associate with OC2 which was labelled with a fluorophore SUN. This brought the fluorophore and the quencher moieties into proximity, causing the fluorophore to be quenched. Since the sensor region of OC1 was designed to be complementary to the target (MG) it could bind to target amplicons during PCR. The extension of the upstream forward primer during PCR caused the sensor region of OC1 to be hydrolysed by the 5′-3′ exonuclease activity of the Taq polymerase, which irreversibly separated the quencher moiety from the rest of the oligonucleotide complex as illustrated schematically in **Figure 7(i)**. The first capture region of OC1 is not bound to the target and hence cannot be hydrolysed by the polymerase. Therefore, the capture region can still bind to OC2 and form a double stranded portion at temperatures below T_m OC1/OC2. The hydrolysis of the sensor region of OC1 caused the fluorophore to fluoresce as it is no longer in proximity to the quencher. In contrast, in the absence of target the M-Tec-H probe complex remains intact and the fluorophore remains quenched as illustrated in **Figure 7(ii)**.

The second temperature, 76°C, was chosen to be above the temperature at which the OC1 and OC2 could hybridize, specifically it was greater than the T_m OC1/OC2. Therefore at 76°C, OC1 and OC2 cannot hybridize and hence the quencher and SUN moieties were not brought into proximity. As a result, there was a constant level of fluorescence which was measured by the thermocycler as background fluorescence which was steady regardless of the presence or absence of target as illustrated schematically in **Figure 7(iii) and (iv)** respectively.

This example demonstrates that M-Tec-H probes allow the target-specific fluorescence signal to be detected at a designated temperature in real-time. The data is consistent with signal generated by hydrolysis of the OC1 within the M-Tec-H probe complex, mediated by the 5'-3' exonuclease activity of the polymerase in a manner similar to that of standard dual labelled TaqMan/Hydrolysis probe. Generation of signal using a protein enzyme to hydrolyse an M-Tec-H probe contrasts with other examples where nucleic acid enzymes were used. Therefore, the chemistry of M-Tec probes is not confined to a single type of enzyme chemistry, but any chemistry that can hydrolyse or cleave the first oligonucleotide component of an M-Tec probe complex in a target-dependent manner could be utilized. The M-Tec-H probe differs from a conventional TaqMan probe in that it can be used for detection of the target at a specific detection temperature (52°C) at a specific wavelength, while showing no change in signal at a higher temperature (76°C) at the same wavelength, regardless of the presence or absence of the target.

Example 5: Real-time detection and quantification of five targets at three wavelengths using a combination of an M-Tec-P probe, a Molecular Beacon, two LOCS probes and a Linear PlexZyme substrate.

The following example demonstrates the combination of an M-Tec-P probe for detection of *Chlamydia trachomatis* (CT), a Molecular Beacon for detection of *Trichomonas vaginalis* (TV), a first LOCS probe for detection of *Mycoplasma genitalium* (MG), a second LOCS probe for detection of a first *Neisseria gonorrhoeae* (NG) gene NGopa and a Linear PlexZyme substrate for detection of a second NG gene, NGporA. Each LOCS probe is paired with either an M-Tec-P probe or Molecular Beacon to allow for simultaneous detection and quantification of two targets in a single fluorescent channel by monitoring fluorescence at two temperatures in real-time during PCR. The M-Tec-P probe, Molecular Beacon and LOCS probe were designed to produce target-

dependent signal at only one temperature, without any crosstalk to the other temperature, whereas the Linear PlexZyme substrate produces target-dependent detectable signal at both temperatures.

Oligonucleotides

The oligonucleotides specific to this experiment include: Forward Primer 1 (SEQ ID: 1), Reverse Primer 1 (SEQ ID: 2), Partzyme A1 (SEQ ID: 3), Partzyme B1 (SEQ ID: 4), OC1/1-Q1 (SEQ ID: 5), OC2/1.1-SUN (SEQ ID: 30), Forward Primer 6 (SEQ ID: 31), Reverse Primer 6 (SEQ ID: 32), Molecular Beacon 2 (SEQ ID: 33), Forward Primer 7 (SEQ ID: 34), Reverse Primer 7 (SEQ ID: 35), Partzyme A6 (SEQ ID: 36), Partzyme B6 (SEQ ID: 37), LOCS-2-HEX/Q1 (SEQ ID: 38), Forward Primer 8 (SEQ ID: 39), Reverse Primer 8 (SEQ ID: 40), Partzyme A7 (SEQ ID: 41), Partzyme B7 (SEQ ID: 42), LOCS-1-FAM/Q1 (SEQ ID: 24), Forward Primer 4 (SEQ ID: 18), Reverse Primer 4 (SEQ ID: 19), Partzyme A8 (SEQ ID: 43), Partzyme B8 (SEQ ID: 44) and Linear-substrate-1-Atto647N/Q2 (SEQ ID: 45).

Reaction conditions

Real-time detection of the target sequence was performed in a total reaction volume of 20 μ L using a QuantStudio™ 7 Pro thermocycler. The cycling parameters were 95°C for 30 seconds, 50 cycles of 95°C for 5 seconds, 52°C for 40 seconds, 61°C for 10 seconds, and 76°C for 18 seconds (data collected at both the 52°C and 76°C steps). All reactions were run in triplicates and contained 40 nM Forward Primer 1, Forward Primer 6, Forward Primer 7, Forward Primer 8 and Forward Primer 4, 200 nM Reverse Primer 1, Partzyme A1, Partzyme B1, OC1/1-Q1, OC2/1.1-SUN, Reverse Primer 7, Partzyme A6, Partzyme B6, LOCS-2-HEX/Q1, Reverse Primer 8, Partzyme A7, Partzyme B7, LOCS-1-FAM/Q1, Reverse Primer 4, Partzyme A8, Partzyme B8 and Linear-substrate-1-Atto647N/Q2, 300 nM Molecular Beacon, 400 nM Reverse Primer 6, 1 $\times$ NH₄ buffer (Meridian Bioscience), 8 mM MgCl₂ (Sigma-Aldrich), 800 μ M dNTP mix (Meridian Bioscience) and 2 units of AptaTaq exo DNA polymerase (Roche CustomBiotech). The reactions either contained no target (NF H₂O), or synthetic double stranded DNA fragments (IDT) homologous to a target gene namely TV or CT or NGopa or NgporA or MG (10000, 1000 or 100 copies) present in a background of 35 ng of human genomic DNA.

Results

During PCR amplification, fluorescence was measured in real-time at two temperatures, where the presence of fluorescence associated with the Molecular Beacon

and/or the M-Tec-P probe was only detected at 52°C when their specific targets were present, and fluorescence associated with each of the two LOCS targets was detected at 76°C when their specific targets were present. The Linear substrate coupled with target-specific PlexZymes gave signal at both acquisition temperatures (**Table 6**).

Table 6: shows the Probe types and their various Fluorophore labels which when read in 3 channels facilitated the detection of five targets indicated as present (+) or absent (-). The table indicates scenarios where Fluorescence (F) above background was observed or where signal contributing to Background (B) only fluorescence was generated.

Channel	Probes (Target detected)	52°C	76°C
VIC	M-Tec Probe – SUN (CT) LOCS Probe – HEX (MG)	+ CT = F - CT = B +/- MG = B	+/- CT = B + MG = F - MG = B
FAM	Molecular Beacon – FAM (TV) LOCS Probe – FAM (Ngopa)	+ TV = F - TV = B +/- Ngopa = B	+/- TV = B + Ngopa = F - Ngopa = B
CY5	Linear PlexZyme Probe – ATTO647 (NgporA)	+ NgporA = F - NgporA = B	+ NgporA = F - NgporA = B

The amplification curves for the reactions described in **Table 6** are shown in **Figure 15**. This single well reaction enabled the detection and discrimination of all five targets using only three fluorescent channels. **Figure 15** shows real time amplification curves generated in a single reaction using a Molecular Beacon (MB) for detection of TV in FAM channel (15A), LOCS probe 1 for detection of Ngopa in FAM channel (15B), M-Tec-P probe (MT) for detection of CT in VIC channel (15C), LOCS probe 2 for detection of MG in VIC channel (15D) and Linear PlexZyme substrate (LS) for detection of NgporA in CY5 Channel (15E), with acquisition at 52°C (52°C; top row) or at 76°C (76°C; bottom row). Reactions contained either 10000 copies of target (a), 1000 copies of target (b) 100 copies of target (c) or no target (d).

In order to achieve the outcome, the melting temperatures of probe components and regions of probes or probe/target hybrids were within the specific design requirements. The relationship of the T_m of components with respect to each other, and/or the target and the acquisition temperature are as defined in **Table 7**.

Table 7: Relationship the T_m 's of Probes and/or probe components or domains and/or target binding regions with respect to acquisition temperatures.

Probe Type	Design requirements for T_m 's of probes and components in relation to acquisition Temp 1 (52°C) and Temp 2 (76°C)	Temp 1 52°C	Temp 2 76°C
M-Tec-P Probe	$T_m \text{ OC1/OC2} > 52^\circ\text{C}$ $T_m \text{ OC1/OC2} < 76^\circ\text{C}$	+ CT = F – CT = B	+/- CT = B
LOCS Probe 1	Intact $T_m \text{ Stem} > 76^\circ\text{C}$ Split $T_m \text{ Stem} > 52^\circ\text{C}$	+/- MG = B	+ MG = F – MG = B
LOCS Probe 2	Split $T_m \text{ Stem} < 76^\circ\text{C}$	+/- NGopa = B	+ NGopa = F – NGopa = B
Molecular Beacon	$T_m \text{ Stem} < T_m \text{ Loop/TV Hybrid}$ $T_m \text{ Stem and Loop/TV Hybrid} > 52^\circ\text{C}$ $T_m \text{ Stem and Loop/TV Hybrid} < 76^\circ\text{C}$	+ TV = F – TV = B	+/- TV = B
Linear PlexZyme Probe	Not Applicable	+ NGporA = F – NGporA = B	+ NGporA = F – NGporA = B

This example illustrates the enormous capability for the approach to provide “mix and match” systems whereby multiple probe types can be combined and manipulated to generate target dependent fluorescence at specific temperatures only. The capacity to generate this target dependent fluorescence from each probe type can be tightly controlled by raising and lowering the temperature allowing switching on and off of probes via association or dissociation of various components, with each other and/or with the target.

Example 6: Detection of a target using an M-Tec-E probe, which generates signal at one temperature only, arising from the nicking endonuclease activity

The following example illustrates an approach whereby an M-Tec-E probe, could be used to detect the presence of a target by monitoring increases in the fluorescence signal at a first temperature, while showing no change in signal at a second higher temperature regardless of the presence or absence of the target.

Figure 16 illustrates an example of an M-Tec-E probe composed of two oligonucleotide components, OC1 and OC2, where OC1 and OC2 have complementary regions with a melting temperature $T_m \text{ OC1/OC2}$ and each is labelled with one detection moiety for example a fluorophore and a quencher. OC1 could be designed to contain a

sensor region complementary to the target, which would also be capable of forming one strand of a double stranded recognition site for a restriction endonuclease, for example a Nicking enzyme.

During a reaction, the fluorescent signal could be monitored at two temperatures. The first temperature could be selected to be below the T_m OC1/OC2, whilst the second temperature could be above the T_m OC1/OC2. At the lower temperature, OC2 would always be hybridized to the capture region of OC1 and intact M-Tec-E probes containing unmodified OC1 would be quenched since the two detection moieties would be held in close proximity.

In the presence of the target, the sensor region of OC1 could hybridize to the target and create a site for the restriction endonuclease. Subsequent cleavage or nicking of the sensor region by the enzyme would cause the formation of a first fragment and a second fragment and separation of the two detection moieties resulting in an increase in the fluorescence signal above the baseline which could be measurable at the first temperature. As such, an increase in fluorescence at the first temperature would indicate the presence of the target. In the absence of target, the OC1 would remain unmodified and intact M-Tec probes would remain quenched.

At the second temperature, OC2 would dissociate from either the cleaved fragments of OC1 generated in the presence of target, or from the intact OC1 present when target is absent. This would result in separation of the detection moieties and contribute to background fluorescence which would be constant and unaffected by the presence or absence of the target.

Specific restriction enzymes may be more favourable for reactions using M-Tec-E probes. M-Tec-E probe could be used to detect unamplified targets or targets which have been amplified using isothermal amplification protocols or PCR. Nicking enzymes could cleave the sensor region of OC1 whilst leaving the target strand, or the target amplicon strand intact and available for subsequent rounds of cleavage of M-Tec-E probes. Thermostable restriction enzymes would be compatible for use with M-Tec-E probes in conjunction with PCR. Reaction conditions which are compatible with PCR amplification and concurrent restriction enzyme cleavage are well known in the art. Further, if the target sequence does not have a convenient region which includes a restriction enzyme site it would be possible to introduce a recognition site which lies partially within the primer and partially within the amplified sequence. Mismatches with respect to the target can be included within the primers such that new restriction sites may be created within

amplicons. Methods for using PCR primers to create amplicons that contain an artificial, induced recognition site for a restriction enzyme are well known in the art.

Example 7: Detection of a target using M-Tec probes composed of more than two oligonucleotides which generates signal at one temperature only

The following example illustrates an approach whereby M-Tec probes, composed of more than two oligonucleotides, could be used to detect the presence of a target by monitoring increases in the fluorescence signal at a first temperature, while causing no change in signal at a second higher temperature regardless of the presence or absence of the target.

M-Tec probes may be composed of at least three oligonucleotides, namely a first oligonucleotide component OC1, a second oligonucleotide component OC2 and a third oligonucleotide component OC3. OC1 could be designed to contain a sensor region that would serve as a substrate that is amenable to modification by an enzyme in the presence of a specific target. The sensor could be flanked by two capture regions, which are complementary to OC2 and OC3, and further OC2 and OC3 could be labelled with a fluorescence dye label at or near the 5' end and a quencher dye label at or near the 3' end, or vice versa.

The fluorescent signal could be monitored at two temperatures. The first "low" temperature could be selected to be below the melting temperature of the complementary regions of both OC1 and OC2 (T_m OC1/OC2), and OC1 and OC3 (T_m OC1/OC3). At this temperature, OC2 and OC3 would always be hybridized to their capture regions of OC1. Intact M-Tec probes containing unmodified OC1 would be quenched since the two dye moieties would be held in close proximity. The second "high" temperature could be selected to be higher than T_m OC1/OC2, or both of the T_m OC1/OC2 and T_m OC1/OC3. As such, at this temperature, either OC2, or both OC2 and OC3, would dissociate from their complementary regions within OC1. Examples of M-Tec-P probes and M-Tec-H probes are illustrated in **Figures 17 and 18** respectively.

Figure 17 illustrates an example of an M-Tec-P probe composed of OC1, OC2 and OC3. Within an M-Tec-P probe, the OC1 could be designed to contain a sensor region that functions as a substrate for a specific PlexZyme, that may be flanked by capture regions which are complementary to OC2 and OC3. Each of OC2 and OC3 could be designed to contain a fluorescence dye label at or near the 5' end or a quencher dye

label at or near the 3' end, or vice versa. None of the three oligonucleotides would contain a sequence related to the target and hence could be universal for any target.

The fluorescent signal could be monitored at two temperatures as defined above. In the presence of the target, the RNA bases in the OC1 oligonucleotide could be cleaved by a PlexZyme designed to assemble in the presence of a specific target. Cleavage of the sensor region OC1 would result in the formation of a first fragment and a second fragment and separation of the two dye moieties would lead to production of target-specific detectable fluorescence signal at the first temperature. In the absence of target, OC1 would not be modified and the two dye labels would remain in close proximity and therefore fluorescence of the intact M-Tec-P probe would be quenched. As such at the first temperature there would be an increase in fluorescence in the presence of target but not in the absence of target.

At the second temperature, OC2 and/or OC3 would dissociate from either the cleaved fragments of OC1 generated in the presence of target, or the intact OC1 present when target is absent. This would result in separation of the dye moieties and contribute to background fluorescence which would be constant and unaffected by the presence or absence of the target.

Figure 18A illustrates an example of an M-Tec-H probe composed of three oligonucleotides, namely OC1, OC2 and OC3. OC1 could be designed to contain a sensor region which is homologous to the target sequence, flanked by two capture regions, each complementary to the OC2 and OC3. Each of OC2 and OC3 could be designed to contain a fluorescence dye label at or near the 5' end or a quencher dye label at or near the 3' end, or vice versa. OC2 and OC3 oligonucleotides would not contain a sequence related to the target and hence all could be universal for any target.

In the presence of the target, during PCR the sensor region of OC1 would be hydrolysed by the 5'-3' exonuclease activity within a polymerase as it would elongate from upstream of where OC1 would be bound on the target. After hydrolysis, the two labels attached to OC2 and OC3 would no longer be held in close proximity and fluorescence signal would be produced. Therefore, increases in the fluorescence signal above the baseline detected at the first temperature would be attributed to the presence of the target. In the absence of target the M-Tec-H probe would remain quenched. At the second temperature, OC2 and/or OC3 would dissociate from either the cleaved fragments of OC1 generated in the presence of target, or from the intact OC1 present when target is absent. This would result in separation of the dye and quencher moieties and contribute to

background fluorescence which would be constant and unaffected by the presence or absence of the target.

Similarly, an M-Tec-E probe (**Figure 18B**) could be composed of three oligonucleotides, namely OC1, OC2 and OC3. OC1 could be designed to contain a sensor region which is homologous to the target sequence, flanked by two capture regions that could be complementary to the OC2 and OC3. The sensor region of OC1 could form one strand of a double stranded recognition site for a restriction endonuclease, for example a Nicking enzyme. OC2 and OC3 could be designed to contain a fluorescence dye label at or near the 5' end and a quencher dye label at or near the 3' end respectively, or vice versa. OC2 and OC3 oligonucleotides would not contain a sequence related to the target and hence could be universal for any target.

In the presence of the target, the sensor region of OC1 could hybridize to the target and complete a recognition site for the restriction endonuclease. Subsequent cleavage or nicking of the sensor region by the enzyme would cause the formation of a first fragment and a second fragment and separation of the dye and quencher moieties on OC2 and OC3 and these would no longer be held in close proximity. Therefore, increases in the fluorescence signal above the baseline detected at the first temperature would be attributed to the presence of the target.

In the absence of target the M-Tec-E probe would remain quenched at the first temperature. At the second temperature, OC2 and/or OC3 would dissociate from either the cleaved fragments of OC1 generated in the presence of target, or from the intact OC1 present when target is absent. This would result in separation of the dye moieties and contribute to background fluorescence which would be constant and unaffected by the presence or absence of the target.

This example illustrates that the composition of M-Tec probes would not be limited to complexes with only two oligonucleotides but could include complexes of at least three oligonucleotide components. One advantage of this design approach would be that the oligonucleotide synthesis may become less complex and less expensive as the number of modifications per oligonucleotide would be reduced. None of the oligonucleotide components which are labelled with dye moieties would be related to a specific target, and therefore the same labelled oligonucleotide components (OC2 and OC3 and/or additional oligonucleotide components) could be universally used for detection of any target. Although this example illustrates compositions for M-Tec probes which comprise three oligonucleotides, it would also be possible to utilize more than three

oligonucleotides whereby at least two are labelled with dye moieties in different locations or orientations to form complexes whereby the two labels would be in proximity at the first temperature. Cleavage or hydrolysis of OC1 by the PlexZyme, or other types of enzymes such as exonuclease or endonuclease could separate the dye moieties leading to target-specific increases in fluorescence at a first temperature. The oligonucleotide components could be designed such that at least one labelled oligonucleotide component could dissociate from the complex at the second higher temperature so that constant contribution to background fluorescence is present which would not be affected by the presence or absence of target.

Example 8: Use of multiple M-Tec probes in a single fluorescent channel to increase the multiplexing capacity

The following example illustrates an approach whereby multiple M-Tec probes labelled with the same dye labels could be utilized to increase the number of targets that could be detected from a single fluorescence channel. A reaction could contain two M-Tec probes, which have the same fluorophore and quencher dye labels, but have different melting temperature for the regions of complementarity between OC1 and OC2 (T_m OC1/OC2). The probes could be designed so that the T_m OC1/OC2 of the first M-Tec probe is lower than that of the second M-Tec probe. M-Tec probes could be designed with two or more oligonucleotides, and the cleavage of OC1 could be mediated by an enzyme in a target-dependent manner, for example by PlexZymes, 5'-3' exonucleases and nicking endonucleases. Some examples of the possible M-Tec probe configurations are illustrated in **Figure 1**, and **Figures 16 to 18**.

An example of two M-Tec-P probes with different T_m OC1/OC2, each composed of two oligonucleotides that could be used in a single reaction is illustrated in **Figure 19**. The first M-Tec-P probe A could be designed to have an OC1 being cleaved during PCR only in the presence of the first target (T1), and a second M-Tec-P probe B could be cleaved only in the presence of the second target (T2). The presence or absence of each of the first and second target could be determined by conducting a melt curve analysis following amplification by PCR. Exemplary melt curve data which could be generated using a pair of M-Tec-P probes as described is illustrated in **Figure 20**. A peak would appear at T_m OC1/OC2 of each probe in the absence of the specific target, for example the M-Tec P probe A with the lower T_m OC1/OC2 would generate the Peak A and the M-Tec P probe B with the higher T_m OC1/OC2 would generate the Peak B. Hence **Figure**

20A would represent an exemplary curve for a "No target control" reaction and/or a reaction containing a sample which does not contain either of the first or second target. **Figure 20B** would represent an exemplary curve for a reaction containing a sample which contains both the first and second targets whilst **Figure 20C** would represent a reaction containing a sample which contains the first but not the second target and **Figure 20D** would represent a reaction containing a sample which contains the second but not the first target. Each of these peaks would not appear in the presence of the specific target because cleavage of OC1 would separate the fluorophore and quencher dye pair and hence a defined peak related to dissociation of OC1 and OC2 fragments would not be observed following PCR when the target was present. Therefore, the presence or absence of each peak would be indicative of the absence or presence of each specific target, respectively.

Alternatively, it would be possible to acquire real-time data during PCR at two different temperatures. At the first temperature below T_m OC1/OC2 of the M-Tec probe A, each probe could generate a signal above the baseline in the presence of their specific targets. In case where both targets are present, both probes would generate signals. At the second temperature above the T_m OC1/OC2 of the M-Tec probe A but below the T_m OC1/OC2 of the M-Tec probe B, the M-Tec probe B would generate a signal above the baseline in the presence of the second target. The M-Tec probe A would generate a constant baseline signal regardless of the presence or absence of the first target and would not contribute to signal above background at the second temperature. Therefore, the detectable signal above the baseline at this temperature would be indicative of the presence of the second target, and the real-time amplification curve could be used to quantify the second target. Using this information from the second temperature, the contribution of the M-Tec probe A in the signal above the baseline at the first temperature could be determined, and consequently the contribution of the M-Tec probe A in signal above the baseline at the first temperature could be elucidated, if present, and therefore the presence of the first target, and quantitative information, could be determined. The detectable signal from the second higher temperature, which relates to the presence or absence of second target only, can be subtracted from the total signal at the first lower temperature, which relates to presence or absence of both the first and second targets, to obtain qualitative or quantitative data relating to the first target only.

Similarly end point data could be collected prior to and following PCR at both the first and second temperatures. An increase in fluorescence observed at the first temperature would indicate the presence of the either first target, or second target, or both.

An increase in fluorescence at the second temperature would indicate the presence of the second target. Using the data acquired at the two temperatures, the contribution of the target specific fluorescence associated with the first target and the M-Tec probe A could be elucidated by subtracting the fluorescence reading at the second temperature from the fluorescence reading at the first temperature.

Although this example outlined the process for designing a reaction for determination of two targets at a single wavelength, it would be possible to extend the multiplexing capacity by using more than two M-Tec probes to be read in the same fluorescent channel. For example, it would be possible to use three M-Tec probes A, B and C where the T_m OC1/OC2 of M-Tec Probe A is less than the T_m OC1/OC2 of M-Tec Probe B which is less than T_m OC1/OC2 of M-Tec Probe C. Use of multiple fluorescent channels could further expand on the multiplexing capacity. Furthermore, it would be possible to include other reporters in the same reaction to be read in the same or different fluorescent channel as the M-Tec probes.

Example 9: Endpoint detection of two targets at a single wavelength using a combination of an M-Tec Probe and a LOCS probe.

The following example demonstrates how the combination of one M-Tec Probe and one LOCS reporter can allow simultaneous detection of two targets in a single fluorescent channel by acquiring fluorescence readings at two temperatures before and after PCR without the need for taking any real-time data acquisitions. Both the M-Tec Probe and the LOCS probe are labelled with the same fluorophore and quencher moieties for simultaneous detection in the same fluorescence channel. This combination of probes was compared to a combination of a Molecular Beacon and a LOCS probe which were also designed to detect the same targets at each temperature in the same way.

The reaction included (i) either an M-Tec-P Probe or a Molecular Beacon which are designed to generate fluorescence above the baseline only in the presence of a first target *Chlamydia trachomatis* (CT) at a first temperature (52°C), but not at 76°C, and (ii) a LOCS reporter which was designed to be cleaved by a second PlexZyme in the presence of a second target *Neisseria gonorrhoeae* (NG) and to generate fluorescence above the baseline at a second temperature (76°C), but not at 52°C. The M-Tec-P Probe was designed be cleaved by a first PlexZyme that is specific to the first target, and the Molecular Beacon was designed to directly bind to the first target sequence in the loop region at the first temperature.

Oligonucleotides

The oligonucleotides specific to this experiment include: Forward Primer 1 (SEQ ID: 1), Reverse Primer 1 (SEQ ID: 2), Partzyme A1 (SEQ ID: 3), Partzyme B1 (SEQ ID: 4), OC1/1-Q1 (SEQ ID: 5), OC2/1-FAM (SEQ ID: 46), Molecular Beacon 2 (SEQ ID: 47), Forward Primer 8 (SEQ ID: 39), Reverse Primer 8 (SEQ ID: 40), Partzyme A7 (SEQ ID: 41), Partzyme B7 (SEQ ID: 42) and LOCS-1-FAM/Q1 (SEQ ID: 24).

Reaction conditions

Amplification and detection of the target sequence was performed in a total reaction volume of 20 μ L using a BioRad® CFX96 thermocycler. The cycling parameters were 95°C for 30 seconds, 52°C for 10 seconds with data acquisition, 76°C for 10 seconds with data acquisition, 45 cycles of 95°C for 1 second and 62°C for 15 seconds, 52°C for 5 minutes with data acquisition, and 76°C for 10 seconds with data acquisition. All reactions were run in triplicates and contained 40 nM of Forward Primers, 200 nM of Reverse Primers, Partzyme A1, Partzyme B1 and LOCS-1-FAM/Q1 oligonucleotides, 1 $\times$ NH₄ buffer (Meridian Bioscience), 8 mM MgCl₂ (Sigma-Aldrich), 800 μ M dNTP mix (Meridian Bioscience) and 2 U AptaTaq exo DNA polymerase (Roche CustomBiotech). The reactions containing the M-Tec probe specifically contained 200 nM of Partzyme A7, Partzyme B7, OC1/1-Q1 and OC2/1-FAM oligonucleotides; the reactions containing the Molecular Beacon specifically contained 200 nM Molecular Beacon 2 oligonucleotide.

The reactions either contained either no target (NF H₂O), or 10000, 800 or 40 copies of synthetic double-stranded DNA fragments (IDT) homologous to the target gene for CT only, or NG only, or both NG and CT, in a background of 35 ng of human genomic DNA (Promega).

Results

Fluorescence was measured in the FAM channel at two temperatures, 52°C and 76°C, at single time points prior to and following 45 cycles of rapid PCR. The presence of a first target (CT) was detected by determining the difference in fluorescence before and after PCR (Δ RFU) at 52°C, and likewise for a second target NG at 76°C. **Figure 21** shows the mean difference in fluorescence before and after PCR for the reactions containing the M-Tec probe and the LOCS probe at 52°C (**21A**) and 76°C (**21B**), and for the reactions containing the Molecular Beacon and the same LOCS probe at 52°C (**21C**) and 76°C (**21D**). The error bars indicate the standard deviation between the replicates. **Figures 21A and 21C** show that the reactions containing either CT alone, or CT mixed

with NG resulted in significantly higher Δ RFU at 52°C than samples that did not contain CT, which is indicative of cleavage of the M-Tec-P probes in the presence of the specific CT target. Similarly, **Figures 21B and 21D** show that the reactions containing either NG alone, or NG mixed with CT, resulted in significantly higher Δ RFU at 76°C compared with samples that do not contain NG, which is indicative of cleavage of the LOCS probes in the presence of the specific NG target.

It is observed in **Figure 21C**, that in the reactions containing a Molecular Beacon, the Δ RFU decreases with the decreasing copy number of CT template in the reaction, while no significant pattern can be observed in **Figure 21A** for the reactions containing an M-Tec-P probe. It is known that some probe types, including Molecular Beacons, can produce endpoint readings or real-time amplification curves wherein the final endpoint fluorescent signals, or the fluorescence level at the plateau phase of amplification curves, decreases as the amount of target template present in the reaction is reduced. In some cases, this may pose a challenge for developing an endpoint qualitative assay, as the decreasing Δ RFU with decreasing target may affect the sensitivity of the assay detrimentally, and with the inconsistent Δ RFU, it may be more difficult to determine an appropriate threshold to differentiate the positive and negative samples. On the other hand, **Figure 21C** shows that the Δ RFU from the M-Tec probe is unaffected by the amount of target in the reaction, and therefore use of this probe may alleviate the potential limitations associated with the use of a Molecular Beacons.

This example demonstrates that qualitative detection of more than one target within a single fluorescent channel can be achieved with the use of an M-Tec-P probe and a LOCS reporter, in a sensitive protocol allowing detection of 40 copies, which showed no cross talk or non-specific signals in off-target reactions. Another advantage of this approach which measures endpoint fluorescence is the possibility of coupling it to rapid PCR cycling with only the requirement for pre- and post-PCR fluorescence measurements. Run times can be significantly decreased compared to real-time protocols which acquire fluorescence during each cycle of PCR. In the experiment in this example the run time, including pre and post fluorescence acquisition, was only 45 minutes. In comparison, the real-time thermocycling protocol used in Example 3 took 108 minutes to complete. As such, endpoint protocols using M-Tec/LOCS probes provide a rapid, and convenient method for facilitating qualitative detection of multiple targets at a single wavelength. This could significantly increase the throughput of sample analysis.

Example 10: Detection of a target using an M-Tec probe and a target-specific Aptazyme

The following example illustrates an approach whereby an M-Tec probe, in combination with a target-specific Aptazyme, could be used to detect the presence of a target by monitoring increases in fluorescence signal at a first temperature, while showing no change in signal at a second higher temperature regardless of the presence or absence of the target. The use of Aptazymes could allow M-Tec probes to detect the presence of a nucleic acid target or a non-nucleic acid target, including proteins, organic molecules or inorganic molecules.

Figure 22 illustrates an example of an M-Tec probe comprising OC1 and OC2 oligonucleotides, and an Aptazyme. The OC1 oligonucleotides could be designed to contain a quencher, a capture region complementary to the sequence of the OC2 oligonucleotide and a sensor region that may function as a substrate for a specific DNzyme. The complementary region between the OC1 and OC2 oligonucleotides could be designed to have a melting temperature being higher than a first temperature, but lower than a second temperature, where the fluorescence measurements would be made. The OC2 oligonucleotide could be designed to contain a fluorophore. The Aptazyme could be designed to contain an aptamer region with a specific affinity to the target, a DNzyme region that could cleave the substrate sequence in the OC1 oligonucleotide, and a cDNA region, which may contain complementary sequences to the aptamer region.

In the absence of the target (**Figure 22A**), the two oligonucleotides of the M-Tec probes could hybridize at the first temperature, as the T_m OC1/OC2 would be higher than this temperature. The two detection moieties, for example fluorophore and quencher moieties, could be brought into proximity and therefore the fluorescence could be quenched. The Aptazyme could form a secondary structure with intramolecular binding between the aptamer and the cDNA regions, and in this conformation the DNzyme could remain inactive.

In the presence of the target (**Figure 22B**), the aptamer region of the Aptazyme would bind to the target, and would not bind to the cDNA region, in which conformation, the DNzyme within the Aptazyme molecule may become active. The active form of DNzyme in the Aptazyme molecule could cleave the sensor region of the OC1 oligonucleotide, the two dye labels in the M-Tec probe could no longer be brought into proximity, which in turn would lead to production of fluorescence signal. Therefore, increases in the fluorescence signal above the baseline detected at the first temperature

could be attributed to the presence of the target. Since the DNAzyme is a multiple-turnover enzyme, even a relatively low concentration of target, and resultant relatively low concentration of active DNAzymes, the Aptazyme could lead to signal production that could be detectable.

At the second temperature that is higher than both the first temperature and T_m OC1/OC2, the OC2 oligonucleotides could not hybridize to the capture region of either the cleaved or intact form of OC1. Therefore, regardless of the OC1 being cleaved or intact, the two labels in the probe complex would not be in proximity, which in turn would contribute to a constant level of fluorescence signal in the presence or absence of the target.

Example 11: Detection of a target using an M-Tec-P probe under isothermal reaction without target amplification.

The following example illustrates an approach whereby an M-Tec-P probe can be used to detect a nucleic acid template which has not been subjected to *in vitro* nucleic acid amplification. The example demonstrates how an M-Tec-P probe can be used for direct detection of a nucleic acid target (*Trichomonas vaginalis*; TV) by running an isothermal reaction at 52°C, without prior amplification of the target. A single stranded DNA fragment which is homologous to TV was incubated for 50 minutes at 52°C in the reaction mix which contained partzymes and an M-Tec-P Probe but did not contain any polymerase enzymes or primers. The endpoint detection of fluorescence signal indicated that fluorescence was generated in the presence, but not in the absence, of the target at a first temperature (52°C), while showing no increase in signal at a second higher temperature (76°C) regardless of the presence or absence of the target.

Oligonucleotides

The oligonucleotides for direct detection of TV specific to this experiment include: Partzyme A9 (SEQ ID: 48), Partzyme B9 (SEQ ID: 49), OC1/1-Q1 (SEQ ID: 5), OC2/1-SUN (SEQ ID: 6). The sequences are listed in the Sequences Listing.

Reaction conditions

Detection of the target sequence was performed in a total reaction volume of 20 µL using a BioRad® CFX96 thermocycler. The temperature parameters were 1 cycle of 52°C for 5 seconds and 76°C for 5 seconds with data acquisition at both temperatures; 1 cycle of isothermal incubation at 52°C for 50 minutes with data acquisition every 30 seconds; 1 cycle of 52°C for 5 seconds and 76°C for 5 seconds with data acquisition at

both temperatures; and finally one melt curve step, where data was acquired between 40°C to 95°C at 0.5°C interval with a hold time of 5 seconds per step. This protocol allowed collection of data in real time during isothermal incubation, at time points pre- and post-isothermal incubation for endpoint analysis, as well as melt curve data post incubation. Fluorescence data was acquired in the VIC Channel. All reactions were run in duplicates and contained 20 nM Partzyme A9, 20 nM Partzyme B9, 200 nM OC1/1-Q1, 200 nM OC2/1-SUN, 1× NH₄ buffer (Meridian Bioscience), and 8 mM MgCl₂ (Sigma-Aldrich).

The reactions either contained no target (NF H₂O), or 125, 25, or 12.5 pM of synthetic single-stranded DNA fragments (IDT), which is homologous to the target gene (TV).

Results

Endpoint detection of the target was performed by measuring the difference between the fluorescence signal acquired before and after the isothermal incubation Δ RFU. The endpoint Δ RFU measurement at 52°C (**Figure 23A**) indicates that there was significant fluorescence signal produced in reactions containing the target DNA but not in reactions where target DNA was absent. The endpoint Δ RFU measurement at 76°C (**Figure 23B**) indicates that there was no increase in signal above background in any reactions regardless of the presence or absence of the target.

Figure 23C shows the real-time fluorescence data plot acquired at 52°C during the isothermal phase of the reaction. The plot shows an increase in fluorescence in real-time in the presence of the target, reaching fluorescence plateau in 10 minutes in the presence of 125 pM (solid grey line) and 25 pM (dashed grey line) of target, and in 25 minutes in the presence of 12.5 pM (dotted grey line) of the target. There was no significant real-time increase in fluorescence for the reactions containing no target template (solid black line).

The derivative melt curve analysis of the reactions (**Figure 24D**) shows melt curve signatures with a peak at 61.5°C for the reactions containing no target (solid black line), which is indicative of intact OC1 within the M-Tec-P probe. This melt signature is absent in the reactions containing the target at all concentrations (solid, dashed or dotted grey line), which is indicative of OC1 cleavage within the M-Tec-P probe. Therefore, the presence or absence of the melt curve signature is indicative of the absence or presence of the target, respectively.

This example demonstrates that M-Tec-P probes may be used for direct detection of nucleic acid targets, where target-dependent signals are selectively produced at a predetermined temperature (52°C), but not at another predetermined temperature (76°C). The identification of signal may be conducted by endpoint detection by taking a single measurement at 52°C after the isothermal step, which could optionally be compared to the measurement taken prior to the isothermal step, real-time measurements at 52°C, or identification of melt curve signatures from the melt curve analysis. The temperature-dependent behaviour of the signal allows for multiplexing potential with different probes that produce signal at different temperatures. This example demonstrates that the signal production mechanism of the M-Tec-P is not directly dependent on the polymerase enzymes or primer extension, and therefore its application could extend to direct detection of the single-stranded target as low as picomolar concentration range. In this example, cleavage of the M-Tec-P probe is mediated by PlexZymes in the presence of target. This example demonstrates an advantage of using M-Tec-P probes for detection of target(s) compared to other protocols which require probe cleavage by the exonuclease activity inherent in polymerase during *in vitro* amplification. Other probe types can only be used in conjunction with *in vitro* amplification wherein primers amplify and the 5'-3' exonuclease activity of the polymerases cleaves a fragment which is essential for the protocols, for example cleavage of TaqMan or other hydrolysis probes or cleavage of Catcher oligonucleotides in variations of the TOCE methodology.

Example 12: Detection of a second target using M-Tec probes which generate target-dependant signal at more than one temperature.

The following example illustrates an approach whereby an M-Tec probe which generates target-dependant detectable signal at both the first and second acquisition temperatures in the presence of a target A may be used in combination with other types of probes, such as Molecular Beacons or TOCE probes, designed to detect Target B at the first acquisition temperature only. Probes could be labelled with the same dye labels which could be detected from a single fluorescence channel. As for other scenarios, the design involves a specific relationship between the melting temperatures of hybridizing regions. For the scenarios in this example the design criteria outlined in Table 8 could be applied.

Table 8: Relation of T_m of components at acquisition Temperature 1 (Temp 1) and acquisition Temperature 2 (Temp 2) in the presence of target (+T) or in the absence of target (-T), Scenarios which could result in increased detectable signal which could be measured as Fluorescence above background (F) or which could contribute to the Background Signal only (B) at Temp 1 and 2 are tabulated. In this example the M-Tec Probe could be designed to detect Target A (TA) and either a Molecular Beacon or a TOCE probe could be designed to detect Target B (TB).

Probe Type	Interrelationship between the T_m 's of probes and components and Temp 1 and 2	Temp 1	Temp 2
M-Tec Probe	$T_m \text{ OC1/OC2} > \text{Temp 1}$ $T_m \text{ OC1/OC2} > \text{Temp 2}$	+ TA = F - TA = B	+ TA = F - TA = B
Molecular Beacon	$T_m \text{ Stem} < T_m \text{ Loop/Target}$ $T_m \text{ Stem and } T_m \text{ Loop/Target} > \text{Temp 1}$ $T_m \text{ Loop/Target} < \text{Temp 2}$	+ TB = F - TB = B	+/- TB = B
TOCE Probe	$T_m \text{ Catcher/Pitcher} > \text{Temp 1}$ $T_m \text{ Catcher/Pitcher} < \text{Temp 2}$	+ TB = F - TB = B	+/- TB = B

A reaction could contain an M-Tec probe and a Molecular Beacon, which have the same fluorophore and quencher dye labels. The M-Tec-P probe could be designed to have an OC1 being cleaved during PCR only in the presence of a first target A. The M-Tec probes could be designed with two or more oligonucleotides, and the cleavage of OC1 could be mediated by an enzyme in a target-dependent manner, for example by a PlexZyme or by the 5'-3' exonuclease activity of a polymerase or by a nicking endonuclease. The M-Tec probe could be designed so that the T_m of OC1/OC2 is above both the first and second acquisition temperatures. The Molecular Beacon could be designed to contain a stem with a T_m that is less than the T_m of the loop/target hybrid, and where the T_m of both the stem and the loop/target hybrid are above the first acquisition temperature but are below the second acquisition temperature. The Molecular Beacon could be designed to hybridise with and detect the presence of a first target B.

It would be possible to acquire real-time data during PCR at two different temperatures. At the first temperature, which is below both the T_m OC1/OC2 of the M-Tec probe and the T_m of the Molecular Beacon's stem and the loop/target hybrid, each probe could generate a signal above the baseline in the presence of their specific targets. In cases where both targets are present, both probes would generate signals at the first

temperature. At the second temperature, which is above the T_m of the Molecular Beacon's stem and the loop/target hybrid but below the T_m OC1/OC2 of the M-Tec probe, only the M-Tec probe would generate a signal above the baseline in the presence of the target A. The Molecular Beacon would generate a constant baseline signal regardless of the presence or absence of the target B and would not contribute to signal above background at the second temperature. Therefore, the detectable signal above the baseline at this temperature would be indicative of the presence of the target A, and the real-time amplification curve could be used to quantify the target A. Using this information from the second temperature, the contribution of the M-Tec probe in the signal above the baseline at the first temperature could be determined, and consequently the contribution of the Molecular Beacon in signal above the baseline at the first temperature could be elucidated, if present, and therefore the presence of the target B, and quantitative information, could be determined.

Similarly, end point data could be collected prior to and following PCR at both the first and second temperatures. An increase in fluorescence observed at the first temperature would indicate the presence of either target A, or target B, or both. An increase in fluorescence at the second temperature would indicate the presence of the target A. Using the data acquired at the two temperatures, the contribution of the target specific fluorescence associated with the target B and the molecular beacon could be elucidated by subtracting the fluorescence reading at the second temperature from the fluorescence reading at the first temperature.

Although this example outlined the process for designing a reaction for determination of two targets at a single wavelength using a combination of Molecular Beacons and M-Tec probes, a skilled person could extend this principle to include other probe types such as TOCE probes. For example, it would be possible to design TOCE probes with the T_m of the Catcher/Pitcher complex above the first acquisition temperature but below the second acquisition temperature and which have the same fluorophore and quencher dye labels. These probes could produce target-dependent signal at only one temperature, without any crosstalk to the other temperature, whereas the abovementioned M-Tec probes would produce target-dependent detectable signal at both temperatures.

This example provides a broad general approach for designing systems for combining probe types that allow detection of targets at specific temperatures only. Further, it provides a wide range of options for detecting multiple targets at a single wavelength.

Sequences

Sequences relevant to the present disclosure are listed in **Table 8**. Sequences are listed from 5' to 3'. UPPERCASE bases represent DNA and lowercase bases represent RNA. /56-FAM/ indicates a FAM fluorophore labelled at 5' end, /5SUN/ indicates a SUN fluorophore labelled at 5' end, /5HEX/ indicates a HEX fluorophore labelled at 5' end, /5Atto647NN/ indicates an Atto 647N fluorophore (NHS ester) labelled at 5' end, /3IB/ represents an Iowa Black FQ quencher capable of absorbing fluorescence in the range of 420-620 nm labelled at 3' end and /3IBR/ represents an Iowa Black RQ quencher used for absorbing fluorescence in the range of 500-700 nm labelled at 3' end. /3Phos/ indicates a 3' phosphate group modification.

Table 9: Sequences used in the Examples

SEQ ID NO	Name	Target	Sequence
SEQ ID NO: 1	Forward Primer 1	<i>Chlamydia trachomatis</i>	AATATCATCTTTGCGGTTGCGTGTCC
SEQ ID NO: 2	Reverse Primer 1	<i>Chlamydia trachomatis</i>	AACCGCTGTGACGGAGTACAAAC
SEQ ID NO: 3	Partzyme A1	<i>Chlamydia trachomatis</i>	TCCTGTGACCTTCATTATGTCGACAA CGAGAGGACTAGG/3Phos/
SEQ ID NO: 4	Partzyme B1	<i>Chlamydia trachomatis</i>	GACGTGAGGAGGCTAGCTGAGTCTGA GCACCCTAGGC/3Phos/
SEQ ID NO: 5	OC1/1-Q1	Universal	ACGCTGTCGATCCGTTACCTAGTCCT CguCCTCACGTC/3IB/
SEQ ID NO: 6	OC2/1-SUN	Universal	/5SUN/TTTTAACGGATCGACAGCGT/3Phos/
SEQ ID NO: 7	Forward Primer 2	<i>Trichomonas vaginalis</i>	GTTTGTGTCTCGTGCCATAGTCG
SEQ ID NO: 8	Reverse Primer 2	<i>Trichomonas vaginalis</i>	ATTTCATGGTCGCCCTCGGAGT
SEQ ID NO: 9	Partzyme A2	<i>Trichomonas vaginalis</i>	TATATGAGTTTGAGACCAAGAATGAC AACGAGAGGTGCGGT/3Phos/
SEQ ID NO: 10	Partzyme B2	<i>Trichomonas vaginalis</i>	AGCTGGGGAGGCTAGCTGTGTAAC GACCTGTCCGATTCA/3Phos/
SEQ ID NO: 11	OC1/2-Q1	Universal	AGGCAGCGACCGGACCGCACCTCguC CCCAGCT/3IB/
SEQ ID NO: 12	OC2/2-FAM	Universal	/56-FAM/AAACCGGTCGCTGCCT/3Phos/
SEQ ID NO: 13	Molecular Beacon 1	<i>Trichomonas vaginalis</i>	/56-FAM/CGGATCAAGAATGGTGTAACTCGACATCCG/3IB/
SEQ ID	Forward	TFRC (<i>Homo</i>	GCTAAAACAATAACTCAGAACTTACG

NO: 14	Primer 3	<i>sapiens</i>)	
SEQ ID NO: 15	Reverse Primer 3	TFRC (<i>Homo sapiens</i>)	CAGCTTTCTGAGGTTACCATCCTA
SEQ ID NO: 16	Partzyme A3	TFRC (<i>Homo sapiens</i>)	GGAATATGGAAGGAGACTGTCACAA CGAGAGGGTCGAG/3Phos/
SEQ ID NO: 17	Partzyme B3	TFRC (<i>Homo sapiens</i>)	GGACGAGGGAGGCTAGCTCCTCTGAC TGGAAAACAGACT/3Phos/
SEQ ID NO: 18	Forward Primer 4	porA (<i>Neisseria gonorrhoea</i>)	GCAGCATTC AATTTGTTCCGAGTC
SEQ ID NO: 19	Reverse Primer 4	porA (<i>Neisseria gonorrhoea</i>)	CGGAAC TGGTTTCATCTGATTACTT
SEQ ID NO: 20	Partzyme A4	porA (<i>Neisseria gonorrhoea</i>)	AAGTCCGCCTATACGCCTGACAACGA GAGGTGCGGT/3Phos/
SEQ ID NO: 21	Partzyme B4	porA (<i>Neisseria gonorrhoea</i>)	AGCTGGGGAGGCTAGCTCTACTTTCA CGCTGGAAAGTA/3Phos/
SEQ ID NO: 22	Partzyme A5	<i>Chlamydia trachomatis</i>	TCCTGTGACCTTCATTATGTCGACAA CGAGAGGGTCGAG/3Phos/
SEQ ID NO: 23	Partzyme B5	<i>Chlamydia trachomatis</i>	GGACGAGGGAGGCTAGCTGAGTCTG AGCACCTAGGC/3Phos/
SEQ ID NO: 24	LOCS-1-FAM/Q1	Universal	/56-FAM/GCGACGGTCTAGAGCTCTTT TCTCGACCCTC _{gu} CCCTCGTCCTTTTG AGCTCTAGACCGTCGC/3IB/
SEQ ID NO: 25	Forward Primer 5	<i>Mycoplasma genitalium</i>	TTTTGTGTTACTAGTGCCTAAGGTGGT AGTGGAGTATCAC
SEQ ID NO: 26	Reverse Primer 5	<i>Mycoplasma genitalium</i>	GGACTCTCTTTTGC AACCTTTGGGTG GG
SEQ ID NO: 27	OC1/3-Q1	<i>Mycoplasma genitalium</i>	GGCGACAGTGC GTTGTAGTCAAAGAG AGTTTGGAAGTGGAAGCA/3IB/
SEQ ID NO: 28	OC2/3-SUN	Universal	/5SUN/AAACGCACTGTCGCC/3Phos/
SEQ ID NO: 29	Aptamer 1	Non-target	GCGATCATCTCAGAACATTCTTAGCG TTTTGTTCTTGTGTATGATCGC/3Phos/
SEQ ID NO: 30	OC2/1.1-SUN	Universal	/5SUN/TAACGGATCGACAGCGT/3Phos/
SEQ ID NO: 31	Forward Primer 6	<i>Trichomonas vaginalis</i>	GAGTCTTTGAATCGGACAGGTGCGAG
SEQ ID NO: 32	Reverse Primer 6	<i>Trichomonas vaginalis</i>	TCGACATGTTTCGTGTCTCGTGCCATA GTCGA
SEQ ID NO: 33	Molecular Beacon 2	<i>Trichomonas vaginalis</i>	/56-FAM/CACCATTCCTTGGTCTCAAAC TCATATGGTG/3IB/
SEQ ID NO: 34	Forward Primer 7	<i>Mycoplasma genitalium</i>	GGCTCGCCATTTAAACCCCTTTGCAC CG
SEQ ID NO: 35	Reverse Primer 7	<i>Mycoplasma genitalium</i>	AATACCTTGATGGTCAGCAAACTTT GCAAT
SEQ ID NO: 36	Partzyme A6	<i>Mycoplasma genitalium</i>	TGCTAAGTTAATATCATATAAAGCTC TAACAACGAGGGACGTCGA/3Phos/
SEQ ID NO: 37	Partzyme B6	<i>Mycoplasma genitalium</i>	GCGGTAGAGGAGGCTAGCTCCGTTGT TATCATACCTTCTGATTG/3Phos/
SEQ ID NO: 38	LOCS-2-HEX/Q1	Universal	/5HEX/GCGACGGTCTAGAGCTCTTTT CGACGTCCC _{gu} CCTCTACCGTTTGTGAG

			CTCTAGACCGTCGC/3IB/
SEQ ID NO: 39	Forward Primer 8	opa (<i>Neisseria gonorrhoea</i>)	ATTGTGTTGAAACACCGCCCGG
SEQ ID NO: 40	Reverse Primer 8	opa (<i>Neisseria gonorrhoea</i>)	GGCTCCTTATTCGGTTTGACCG
SEQ ID NO: 41	Partzyme A7	opa (<i>Neisseria gonorrhoea</i>)	GGAACCCGATATAATCCGCACAACG AGAGGGTCGA/3Phos/
SEQ ID NO: 42	Partzyme B7	opa (<i>Neisseria gonorrhoea</i>)	GGACGAGGGAGGCTAGCTCCTTCAAC ATCAGTGAAAATC/3Phos/
SEQ ID NO: 43	Partzyme A8	porA (<i>Neisseria gonorrhoea</i>)	AAGTCCGCCTATACGCCTGACAACGA GGGGAGAGGA/3phos/
SEQ ID NO: 44	Partzyme B8	porA (<i>Neisseria gonorrhoea</i>)	TTGAAGGGGAGGCTAGCTCTACTTTC ACGCTGGAAAGTA/3Phos/
SEQ ID NO: 45	Linear- substrate- 1- Atto647N/ Q2	Universal	/5ATTO647NN/TCCTCTCCCCguCCCCT TCAA/3IBR/
SEQ ID NO: 46	OC2/1- FAM	Universal	/56-FAM/GCGTAACGGATCGACAGCGT /3Phos/
SEQ ID NO: 47	Molecular Beacon 2	<i>Chlamydia trachomatis</i>	/56-FAM/CCTCACGTCATTATGTGCGGA GTCTGAGCACCGTGAGG/3IABkFQ/
SEQ ID NO: 48	Partzyme A9	<i>Trichomonas vaginalis</i>	AAGCTCACAACACCAACATACGACA ACGAGAGGACTAGG/3Phos/
SEQ ID NO: 49	Partzyme B9	<i>Trichomonas vaginalis</i>	GACGTGAGGAGGCTAGCTGCGATCTT AACCACCTTGTTTC/3Phos/

CLAIMS

1. A method for determining the presence or absence of a target in a sample, the method comprising:

(a) preparing a mixture for a reaction by contacting the sample or a derivative thereof putatively comprising the target with:

- a multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,

wherein the first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a first double-stranded portion,

wherein the first oligonucleotide component further comprises a sensor region capable of serving as a substrate for an enzyme,

wherein the first oligonucleotide component is connected to a first detection moiety and the second oligonucleotide component is connected to a second detection moiety;

- an enzyme capable of modifying the sensor region of the first oligonucleotide component only when the target is present in the sample;

(b) treating the mixture under conditions suitable for the enzyme to modify the sensor region of the first oligonucleotide component to thereby generate a first fragment comprising the first capture region and a second fragment connected to the first detection moiety, thereby enabling the first and second detection moieties to spatially separate and generate a first detectable signal,

(c) measuring a level of background signal or detectable signal generated at a defined temperature at or below which the first capture region is hybridised to the second oligonucleotide component; and

(d) determining the presence or absence of the target based upon the level of detectable signal measured at the defined temperature, wherein a detectable signal at the defined temperature is indicative of the presence of the target in the sample.

2. The method of claim 1 wherein the enzyme is capable of digesting the sensor region of the first oligonucleotide component only when the target is present in the sample, and wherein step (b) comprises treating the mixture under conditions suitable for the enzyme to digest the sensor region of the first oligonucleotide component to thereby generate a

first fragment comprising the first capture region and a second fragment connected to the first detection moiety.

3. The method of claim 1 or claim 2 wherein the method comprises:

(i) measuring a level of background signal or detectable signal generated by the first and second detection moieties in the mixture at the defined temperature

- at a timepoint prior to or during said treating the mixture, and
- at one or more subsequent timepoint(s) during or following said treating the mixture; and

(ii) determining a presence of or a change in the level of detectable signal which differs from the background signal and is indicative of the presence of the target in the sample.

4. The method of claim 3 wherein step (c) comprises measuring the detectable signal and/or any said background signal:

- at one or more timepoints prior to said treating;
- at one or more timepoints during said treating;
- at one or more timepoints after said treating;
- at one or more timepoints during said treating and at one or more timepoints after said treating;
- at one or more timepoints prior to said treating and at one or more timepoints after said treating; or
- at one or more timepoints before and during said treating and at one or more timepoints after said treating.

5. The method of claim 3 or claim 4 wherein step (d) comprises using a predetermined threshold value to determine if the detectable signal differs from any said background signal at the defined temperature.

6. The method of claim 1 or claim 2 further comprising measuring a level of control background signal generated at the defined temperature in a control mix,

and wherein step (c) comprises measuring a level of the background or detectable signal in the mixture contacted by the sample or derivative thereof,

and wherein step (d) comprises determining whether a detectable signal that differs from the control background signal is generated and indicative of the presence of the target in the sample.

7. The method of claim 1 or claim 2 further comprising:

measuring a level of control background signal generated at the defined temperature in a control mix, and

determining whether the level of control background signal measured in the control mix differs from the level of background signal or detectable signal measured in the mixture at step (c),

wherein a difference in the level of background signal or detectable signal measured in the mixture at step (c) compared to the level of control background signal measured in the control mix is indicative of the presence of the target in the sample.

8. The method of claim 6 or claim 7 wherein the control mix does not comprise the target but is otherwise equivalent to the mixture.

9. The method of claim 6 or claim 7 wherein the control mix does not comprise the enzyme but is otherwise equivalent to the mixture.

10. The method of claim 1 or claim 2 further comprising:

measuring a level of control detectable signal generated at the defined temperature in a control mix, wherein the control mix comprises a predetermined amount of the target but is otherwise equivalent to the mixture; and

determining whether the level of control detectable signal measured in the control mix differs from the level of background signal or detectable signal measured in the mixture at step (c),

wherein a difference in the level of background signal or detectable signal measured in the mixture at step (c) compared to the level of control detectable signal measured in the control mix is indicative of the presence and/or amount of the target in the sample.

11. The method of any one of claims 1 to 10 wherein the target is a nucleic acid and at least a portion of the sensor region hybridises to a complementary sequence in the target to thereby form a duplex between the sensor region and the target.

12. The method of claim 11 wherein the enzyme is an endonuclease that recognises a sequence in the duplex.

13. The method of claim 12 wherein the endonuclease digests at least one strand of the duplex to thereby form the first and second fragments.

14. The method of claim 12 wherein the endonuclease is a nicking endonuclease that digests the sensor region of the first oligonucleotide component after formation of the duplex to thereby form the first and second fragments.

15. The method of claim 11 wherein the enzyme is an exonuclease that hydrolyses the sensor region of the first oligonucleotide component after formation of the duplex to thereby form the first and second fragments.

16. The method of claim 15 wherein the exonuclease is a polymerase with exonuclease activity.

17. The method of claim 16 wherein

- the target is a nucleic acid,
- at least a portion of the sensor region hybridises to a complementary sequence in the target to thereby form a duplex between the sensor region and the target,
- said mixture further comprises a target primer capable of binding to the target at a region upstream of said complementary sequence,
- said treating the mixture comprises:
 - hybridisation of the target primer to the target by complementary base pairing,
 - extending the primer using the polymerase with exonuclease activity and using the nucleic acid target as a template,
 - wherein the polymerase comprising exonuclease activity digests the sensor region of the first oligonucleotide component after formation of the duplex to thereby form the first and second fragments.

18. The method of any one of claims 1 to 10 wherein the enzyme is a DNAzyme.

19. The method of any one of claims 1 to 10 wherein the target is a nucleic acid and the sensor region of the first oligonucleotide component is not complementary to the target.

20. The method of any one of claims 1 to 10 or 19 wherein the target is a nucleic acid and the enzyme is a multi-component nucleic acid enzyme (MNAzyme) comprising two partzyme oligonucleotides capable of self-assembling to form the MNAzyme only in the presence of the target.

21. The method of claim 20 wherein said treating comprises:

hybridising sensor arms of the MNAzyme to the target by complementary base pairing, and

hybridising substrate arms of the MNAzyme to at least a portion of the sensor region of the first oligonucleotide component by complementary base pairing to facilitate cleavage of the first oligonucleotide component and generation of the first and second fragments.

22. The method of any one of claims 1 to 21 wherein the target is a nucleic acid.

23. The method of claim 22 wherein the target is an amplicon of a nucleic acid.

24. The method of claim 23 wherein the amplicon is produced by an amplification reaction selected from the group consisting of polymerase chain reaction (PCR), strand displacement amplification (SDA), nicking enzyme amplification reaction (NEAR), helicase dependent amplification (HDA), Recombinase Polymerase Amplification (RPA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), transcription-mediated amplification (TMA), self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), Ligase Chain Reaction (LCR) or Ramification Amplification Method (RAM) and reverse transcription polymerase chain reaction (RT-PCR).

25. The method of claim 24, wherein said detecting:

- occurs prior to said amplification or within 1, 2, 3, 4, or 5 cycles of said amplification commencing; and/or

- occurs after completion of said amplification.

26. The method of any one of claims 23 to 25 wherein said determining the presence or absence of the target comprises a melt curve analysis.

27. The method of any one of claims 1 to 10 wherein:

- the enzyme is a DNAzyme or a ribozyme requiring a co-factor for catalytic activity,
 - said treating of the mixture comprises using conditions suitable for:
 - binding of the cofactor to the DNAzyme or ribozyme to render it catalytically active,
 - hybridisation of the DNAzyme or ribozyme to the first oligonucleotide component by complementary base pairing,
 - catalytic activity of the DNAzyme or ribozyme to thereby digest the first oligonucleotide component and generate the first fragment and the second fragment, and
 - the target is the co-factor.

28. The method of claim 27 wherein the co-factor is a metal ion, such as a metal ion selected from: Mg^{2+} , Mn^{2+} , Ca^{2+} and Pb^{2+} .

29. The method of any one of claims 1 to 10 wherein the enzyme is an aptazyme wherein:

- the sensor region comprises a substrate for an aptazyme;
- the target is an analyte, protein, peptide, compound or nucleic acid;
- the mixture comprises an aptazyme comprising an aptamer capable of binding to the target; and
 - said treating the mixture further comprises binding of the aptazyme to the target and to the sensor region to facilitate cleavage of the first oligonucleotide component to thereby generate the first fragment and the second fragment.

30. A method for determining the presence or absence of a target in a sample, the method comprising:

- (a) preparing a mixture for a reaction by contacting the sample or a derivative thereof putatively comprising the target with:

- a multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,
 - wherein the first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a first double-stranded portion,
 - wherein the first oligonucleotide component further comprises a sensor region capable of serving as a substrate for an enzyme,
 - wherein the first oligonucleotide component is connected to a first detection moiety and the second oligonucleotide component is connected to a second detection moiety;
- an enzyme capable of modifying the sensor region of the first oligonucleotide component only when the target is present in the sample;
- (b) treating the mixture under conditions suitable for the enzyme to modify the sensor region of the first oligonucleotide component to thereby generate a first fragment comprising the first capture region and a second fragment connected to the first detection moiety, thereby enabling the first and second detection moieties to spatially separate and generate a first detectable signal,
- (c) measuring a level of detectable signal generated at a plurality of temperatures including at temperatures below and above a temperature at which the first capture region hybridises to the second oligonucleotide component; and
- (d) determining the presence or absence of the target based upon the presence or absence of a change in detectable signal generating a melt curve peak, wherein absence of a melt curve peak is indicative of the presence of the target in the sample and presence of a melt curve peak is indicative of the absence of the target in the sample.

31. The method of any one of claims 1 to 30 wherein the second oligonucleotide component is directly labelled with the second detection moiety.

32. The method of any one of claims 1 to 31 wherein the first fragment is not directly labelled with a detection moiety.

33. The method of any one of claims 1 to 32 wherein the second fragment is not directly labelled with the first detection moiety.

34. The method of any one of claims 1 to 33 wherein the first oligonucleotide component is not directly labelled with the first detection moiety.

35. The method of any one of claims 1 to 34 wherein the first oligonucleotide component is connected to the first detection moiety via a third oligonucleotide component, wherein
the first oligonucleotide component further comprises a second capture region capable of hybridisation to the third oligonucleotide component by complementary base pairing to form a second double-stranded portion, and
the third oligonucleotide component is directly labelled with the first detection moiety.

36. The method of claim 35 wherein the first double-stranded portion and the second double-stranded portion of the multi-component temperature-controlled probe have a melting temperature (T_m) that is above the defined temperature.

37. The method of claim 35 or claim 36 wherein the T_m of the first double-stranded portion is less than the T_m of the second double-stranded portion.

38. The method of claim 35 or claim 36 wherein the T_m of the second double-stranded portion is less than the T_m of the first double-stranded portion.

39. The method of any one of claims 1 to 31 wherein the first oligonucleotide component is directly labelled with the first detection moiety.

40. The method of any one of claims 1 to 39 wherein the multi-component temperature-controlled probe does not comprise more than two detection moieties.

41. The method of any one of claims 1 to 40 wherein the first oligonucleotide component is not directly labelled with more than one detection moiety.

42. The method of any one of claims 1 to 41 wherein the first oligonucleotide component is not connected to more than one detection moiety.

43. The method of any one of claims 1 to 42 wherein the second oligonucleotide component is not directly labelled with more than one detection moiety.

44. The method of any one of claims 1 to 43 wherein the second oligonucleotide component is not connected to more than one detection moiety.

45. The method of any one of claims 1 to 44 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore.

46. The method of any one of claims 1 to 45 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore;

and wherein the multi-component temperature-controlled probe does not comprise more than one quencher.

47. The method of any one of claims 1 to 46 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore;

and wherein the detectable signal is fluorescence emitted in the presence of the target.

48. The method of any one of claims 1 to 47 wherein neither the first oligonucleotide component, the second oligonucleotide component nor the third oligonucleotide component serve as a primer for a DNA polymerase in an extension reaction.

49. The method of any one of claims 1 to 48 wherein neither the first oligonucleotide component, the second oligonucleotide component nor the third oligonucleotide component serve as a template for a DNA polymerase in an extension reaction.
50. The method of any one of claims 1 to 49 wherein neither the second oligonucleotide component nor the third oligonucleotide component is enzymatically cleaved or degraded.
51. The method of any one of claims 1 to 50 wherein the sensor region is located between the first capture region and the first detection moiety.
52. The method of any one of claims 1 to 51 wherein following said treating the mixture the first fragment is capable of hybridizing to the second oligonucleotide component via the first capture region.
53. The method of any one of claims 1 to 52 wherein the biological sample is obtained from a subject.
54. The method of any one of claims 1 to 53 wherein generation of the detectable signal at the defined temperature is not reversible.
55. The method of any one of claims 1 to 54 wherein the method is performed *in vitro*.
56. The method of any one of claims 1 to 54 wherein the method is performed *ex vivo*.
57. A method for determining the presence or absence of a first target and a second target in a sample, the method comprising:
- (a) preparing a mixture for a reaction by contacting the sample or a derivative thereof putatively comprising the first and/or second target with:
 - a multi-component temperature-controlled probe for detection of the first target, the multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,
 - wherein the first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a first double-stranded portion,

wherein the first oligonucleotide component further comprises a sensor region capable of serving as a substrate for an enzyme,

wherein the first oligonucleotide component is connected to a first detection moiety and the second oligonucleotide component is connected to a second detection moiety,

- a second nucleic acid probe for detection of the second target, the second nucleic acid probe comprising third and fourth detection moieties,

wherein the first and second detection moieties are capable of generating a first detectable signal, and the third and fourth detection moieties are capable of generating a second detectable signal, and

- a first enzyme capable of modifying the sensor region of the first oligonucleotide component only when the first target is present in the sample;

(b) treating the mixture under conditions suitable for:

- the first enzyme to modify the sensor region of the first oligonucleotide component to thereby generate a first fragment comprising the first capture region and a second fragment connected to the first detection moiety, thereby enabling the first and second detection moieties to spatially separate and generate a first detectable signal,

- the second target to induce a modification of the second nucleic acid probe, thereby enabling the third and fourth detection moieties to spatially separate and generate a second detectable signal;

(c) measuring a level of background or detectable signal:

- at a first temperature at or below which the first capture region is hybridised to the second oligonucleotide component,

- at a second temperature at or above which the first capture region is not hybridised to the second oligonucleotide component,

(d) determining whether at one or more timepoints during or after said treating:

- a first detectable signal is generated at the first temperature at or below which the first capture region is hybridised to the second oligonucleotide component,

- a second detectable signal arising from said modification of the second nucleic acid probe is generated at the second temperature, wherein the second detectable signal is indicative of the presence of the second target in the sample.

58. The method of claim 57 wherein the first enzyme is capable of digesting the sensor region of the first oligonucleotide component only when the first target is present in the

sample, and wherein step (b) comprises treating the mixture under conditions suitable for the first enzyme to digest the sensor region of the first oligonucleotide component to thereby generate a first fragment comprising the first capture region and a second fragment connected to the first detection moiety.

59. The method of claim 57 or claim 58 wherein a first detectable signal at the first temperature is indicative of the presence of the first target in the sample.

60. The method of claim 59 wherein the presence of the first target is determined at the first temperature based upon the first detectable signal generated at the first temperature.

61. The method of any one of claims 57 to 60 wherein the presence of the second target is determined at the second temperature based upon the second detectable signal generated at the second temperature.

62. The method of claim 57 or claim 58 wherein:

(i) at the first temperature

- a first detectable signal is generated in the presence of the first target,
- a second detectable signal is generated in the presence of the second target, or
- a first detectable signal and a second detectable signal is generated in the

presence of both the first target and the second target; and

(ii) a second detectable signal is generated at the second temperature only in the presence of the second target.

63. The method of claim 62 wherein the presence of the first target is determined by subtracting any second detectable signal detected at the second temperature from any first and/or second detectable signal detected at the first temperature.

64. The method of any one of claims 57 to 63 wherein the method comprises:

- measuring a level of background signal or detectable signal at the first and second temperatures generated by the first and second detection moieties and by the third and fourth detection moieties in the mixture,

- determining a presence of or a change in the level of the first detectable signal which differs from the background signal and is indicative of the presence of the first target in the sample, and
- determining a presence of or a change in the level of the second detectable signal arising from said modification generated at the second temperature which differs from the background signal and is indicative of the presence of the second target in the sample.

65. The method of any one of claims 57 to 64 wherein at the second temperature, dissociation of the second oligonucleotide component from the capture region of either the first oligonucleotide component present in the absence of the first target, or the first fragment generated by modification of the first oligonucleotide component in the presence of the first target generate an equal, similar or equivalent background signal.

66. The method of any one of claims 57 to 65 wherein said determining comprises detection of the first detectable signal and/or any said background signal:

- at one or more timepoints prior to said treating
- at one or more timepoints during said treating;
- at one or more timepoints after said treating;
- at one or more timepoints during said treating and at one or more timepoints after said treating;
- at one or more timepoints prior to said treating and at one or more timepoints after said treating; or
- at one or more timepoints before and during said treating and at one or more timepoints after said treating.

67. The method of any one of claims 57 to 66, wherein said determining in part (d) comprises:

- using a predetermined threshold value to determine if the first detectable signal differs from any said background signal at the first temperature; and/or
- using a predetermined threshold value to determine if the second detectable signal differs from any said background signal at the second temperature.

68. The method of any one of claims 57 to 63 comprising:

measuring a level of first control background signal at the first temperature provided by the first and second detection moieties and by the third and fourth detection moieties in a control mix;

measuring a level of second control background signal at the second temperature provided by the first and second detection moieties and by the third and fourth detection moieties in the control mix;

determining whether a level of the first detectable signal generated at the first temperature at step (c) in the mixture contacted by the sample or derivative thereof differs from the level of first control background signal measured in the control mix, wherein a difference in the level of detectable signal measured in the mixture at the first temperature at step (c) compared to the first control background signal measured in the control mix is indicative of the first target in the sample; and

determining whether a level of the second detectable signal generated at the second temperature at step (c) in the mixture contacted by the sample or derivative thereof differs from the level of second control background signal measured in the control mix, wherein a difference in the level of detectable signal measured in the mixture at the second temperature at step (c) compared to the second control background signal measured in the control mix is indicative of the second target in the sample.

69. The method of claim 68 wherein the control mix does not comprise:

- the first target;
- the second target; or
- the first and second targets,

but is otherwise equivalent to the mixture.

70. The method of claim 68 wherein the control mix does not comprise the first enzyme but is otherwise equivalent to the mixture.

71. The method of any one of claims 57 to 63 further comprising:

measuring a level of first control detectable signal generated at the first temperature in a control mix, wherein the control mix comprises a predetermined amount of

- the first target,
- the second target, or
- the first and second targets,

but is otherwise equivalent to the mixture;

measuring a level of second control detectable signal generated at the second temperature in the control mix;

determining whether a level of the first detectable signal generated at the first temperature at step (c) in the mixture contacted by the sample or derivative thereof differs from the level of first control detectable signal measured in the control mix, wherein a difference in the level of detectable signal measured in the mixture at the first temperature at step (c) compared to the first control detectable signal measured in the control mix is indicative of the first target in the sample; and

determining whether a level of the second detectable signal generated at the second temperature at step (c) in the mixture contacted by the sample or derivative thereof differs from the level of second control detectable signal measured in the control mix, wherein a difference in the level of detectable signal measured in the mixture at the second temperature at step (c) compared to the second control detectable signal measured in the control mix is indicative of the second target in the sample.

72. The method of any one of claims 57 to 67 wherein part (c) comprises measuring a first background signal at or within 1°C, 2°C, 3°C, 4°C or 5°C of the first temperature, and a second background signal at or within 1°C, 2°C, 3°C, 4°C or 5°C of the second temperature.

73. The method of claim 72 wherein part (d) comprises determining whether at one or more time points during or after said treating:

a first detectable signal is generated at the first temperature which differs from the first background signal and is indicative of the presence of the first target in the sample; and

a second detectable signal is generated at the second temperature which differs from the second background signal and is indicative of the presence of the second target in the sample.

74. The method of any one of claims 57 to 73 wherein at the first temperature the third and fourth detection moieties do not generate a signal which differs from the background signal.

75. The method of any one of claims 57 to 74 wherein at the second temperature the first and second detection moieties do not generate a signal which differs from the background signal.

76. The method of any one of claims 57 to 75 wherein the first and second detectable signals are detectable by a single detector.

77. The method of any one of claims 57 to 76 wherein the first and second detectable signals are detectable in the same fluorescent channel.

78. The method of any one of claims 57 to 77 wherein the first and second detectable signals are detectable as fluorescent emission at a single wavelength.

79. The method of any one of claims 57 to 78, wherein the first and second detection moieties, and the third and fourth detection moieties emit a detectable signal at the same or similar wavelength which can be detected in the same fluorescence channel.

80. The method of any one of claims 57 to 79 wherein the second oligonucleotide component is directly labelled with the second detection moiety.

81. The method of any one of claims 57 to 80 wherein the first fragment is not directly labelled with a detection moiety.

82. The method of any one of claims 57 to 81 wherein the second fragment is not directly labelled with the first detection moiety.

83. The method of any one of claims 57 to 82 wherein the first oligonucleotide component is not directly labelled with the first detection moiety.

84. The method of any one of claims 57 to 83 wherein the first oligonucleotide component is connected to the first detection moiety via a third oligonucleotide component, wherein

the first oligonucleotide component further comprises a second capture region capable of hybridisation to the third oligonucleotide component by complementary base pairing to form a second double-stranded portion, and

the third oligonucleotide component is directly labelled with the first detection moiety.

85. The method of claim 84 wherein the first double-stranded portion and the second double-stranded portion of the multi-component temperature-controlled probe have a T_m that is above the first temperature.

86. The method of claim 84 or claim 85 wherein the first double-stranded portion and/or the second double-stranded portion of the multi-component temperature-controlled probe have a T_m that is below the second temperature.

87. The method of any one of claims 84 to 86 wherein the T_m of the first double-stranded portion is less than the T_m of the second double-stranded portion.

88. The method of any one of claims 84 to 86 wherein the T_m of the second double-stranded portion is less than the T_m of the first double-stranded portion.

89. The method of any one of claims 57 to 80 wherein the first oligonucleotide component is directly labelled with the first detection moiety.

90. The method of any one of claims 57 to 89 wherein the multi-component temperature-controlled probe does not comprise more than two detection moieties.

91. The method of any one of claims 57 to 90 wherein the first oligonucleotide component is not directly labelled with more than one detection moiety.

92. The method of any one of claims 57 to 91 wherein the first oligonucleotide component is not connected to more than one detection moiety.

93. The method of any one of claims 57 to 92 wherein the second oligonucleotide component is not directly labelled with more than one detection moiety.

94. The method of any one of claims 57 to 93 wherein the second oligonucleotide component is not connected to more than one detection moiety.

95. The method of any one of claims 57 to 94 wherein the second nucleic acid probe is directly labelled with the third and fourth detection moieties.

96. The method of any one of claims 57 to 94 wherein the second nucleic acid probe is not directly labelled with the third and fourth detection moieties.

97. The method of any one of claims 57 to 96 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore.

98. The method of any one of claims 57 to 97 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore;

and wherein the multi-component temperature-controlled probe does not comprise more than one quencher.

99. The method of any one of claim 57 to 98 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore;

and wherein the first detectable signal is fluorescence emitted in the presence of the first target.

100. The method of any one of claims 57 to 99 wherein neither the first oligonucleotide component, the second oligonucleotide component nor the third oligonucleotide component serve as a primer for a DNA polymerase in an extension reaction.

101. The method of any one of claims 57 to 100 wherein neither the first oligonucleotide component, the second oligonucleotide component nor the third oligonucleotide component serve as a template for a DNA polymerase in an extension reaction.

102. The method of any one of claims 57 to 101 wherein neither the second oligonucleotide component nor the third oligonucleotide component is enzymatically cleaved or degraded.

103. The method of any one of claims 57 to 102 wherein the sensor region is located between the first capture region and the first detection moiety.

104. The method of any one of claims 57 to 103 wherein following said treating the first fragment is capable of hybridizing to the second oligonucleotide component via the first capture region.

105. The method of any one of claims 57 to 104 wherein:

the third detection moiety is a fluorophore, and the fourth detection moiety is a quencher; or

the third detection moiety is a quencher, and the fourth detection moiety is a fluorophore.

106. The method of any one of claims 57 to 105 wherein the first target is a nucleic acid and at least a portion of the sensor region hybridises to a complementary sequence in the first target to thereby form a duplex between the sensor region and the first target.

107. The method of claim 106 wherein the first enzyme is an endonuclease that recognises a sequence in the duplex.

108. The method of claim 107 wherein the endonuclease digests the duplex to thereby form the first and second fragments.

109. The method of claim 107 wherein the endonuclease is a nicking endonuclease that digests the sensor region of the first oligonucleotide component after formation of the duplex to thereby form the first and second fragments.

110. The method of claim 106 wherein the first enzyme is an exonuclease that hydrolyses the sensor region of the first oligonucleotide component after formation of the duplex to thereby form the first and second fragments.

111. The method of claim 110 wherein the exonuclease is a polymerase with exonuclease activity.

112. The method of claim 111 wherein

- the first target is a nucleic acid,
- at least a portion of the sensor region hybridises to a complementary sequence in the first target to thereby form a duplex between the sensor region and the first target,
- said mixture further comprises a first target primer capable of binding to the first target at a region upstream of said complementary sequence,
- said treating the mixture comprises:
 - hybridisation of the first target primer to the first target by complementary base pairing,
 - extending the primer using the polymerase with exonuclease activity and using the first target as a template,

wherein the polymerase comprising exonuclease activity digests the sensor region of the first oligonucleotide component after formation of the duplex.

113. The method of any one of claims 57 to 105 wherein the first enzyme is a DNAzyme.

114. The method of any one of claims 57 to 105 wherein the first target is a nucleic acid and the sensor region of the first oligonucleotide component is not complementary to the first target.

115. The method of any one of claims 57 to 105 or 114 wherein the first target is a nucleic acid and the first enzyme is a first target multi-component nucleic acid enzyme

(MNAzyme) comprising two partzyme oligonucleotides capable of self-assembling to form the first target MNAzyme only in the presence of the first target.

116. The method of claim 115 wherein said treating comprises:

hybridising sensor arms of the first target MNAzyme to the first target by complementary base pairing, and

hybridising substrate arms of the first target MNAzyme to at least a portion of the sensor region of the first oligonucleotide component by complementary base pairing to facilitate cleavage of the first oligonucleotide component and generation of the first and second fragments.

117. The method of any one of claims 57 to 116 wherein:

- the second nucleic acid probe is a substrate for a second target multi-component nucleic acid enzyme (MNAzyme) the second target MNAzyme comprising two partzyme oligonucleotides capable of self-assembling to form the second target MNAzyme only in the presence of the second target;

- the mixture further comprises:

the second target MNAzyme is capable of cleaving the second nucleic acid probe only when the second target is present in the sample;

- said treating further comprises:

hybridising sensor arms of the second target MNAzyme to the second target by complementary base pairing, and

hybridising substrate arms of the second target MNAzyme to the second nucleic acid probe by complementary base pairing to facilitate cleavage of the second nucleic acid probe thereby providing said modification to the second nucleic acid probe and enabling the third and fourth detection moieties to spatially separate and generate the second detectable signal.

118. The method of claim 117 wherein the second nucleic acid probe is a stem-loop oligonucleotide comprising a double-stranded stem portion of hybridised nucleotides opposing strands of which are linked by an unbroken single-stranded loop portion of unhybridised nucleotides of which all or a portion is complementary to the substrate arms of the second target MNAzyme.

119. The method of claim 118 wherein the stem-loop oligonucleotide is an intact stem-loop oligonucleotide and the said modification comprises cleavage of the loop portion and the formation of a split stem-loop oligonucleotide.

120. The method of any one of claims 57 to 116 wherein:

- the second target is a nucleic acid,
- the second nucleic acid probe is a stem-loop oligonucleotide comprising a double-stranded stem portion of hybridised nucleotides opposing strands of which are linked by an unbroken single-stranded loop portion of unhybridised nucleotides of which all or a portion is complementary to the second target,
- the mixture further comprises a polymerase with exonuclease activity,
- said treating the mixture comprises using conditions suitable for:
 - hybridisation of the second target to the single-stranded loop portion of the stem-loop oligonucleotide by complementary base pairing to form a first double-stranded sequence comprising a portion of the second target,
 - hybridisation of a primer to the second target to form a second double-stranded sequence located upstream relative to the first double-stranded sequence comprising the portion of the second target,
 - extending the primer using the polymerase with exonuclease activity and using the second target as a template,
 - wherein the polymerase comprising exonuclease activity digests the single-stranded loop portion of the first double-stranded sequence and thereby forms a split stem-loop oligonucleotide.

121. The method of any one of claims 57 to 116, wherein:

- the second target is a nucleic acid,
- the second nucleic acid probe is a stem-loop oligonucleotide comprising a double-stranded stem portion of hybridised nucleotides opposing strands of which are linked by an unbroken single-stranded loop portion of unhybridised nucleotides of which all or a portion is complementary to the second target,
- the mixture further comprises an endonuclease, and
- said treating the mixture comprises using conditions suitable for:

hybridisation of the second target to the single-stranded loop portion of the stem-loop oligonucleotide by complementary base pairing to form a double-stranded sequence comprising a portion of the second target,

association of the endonuclease with the double-stranded sequence comprising a portion of the second target, and

catalytic activity of endonuclease allowing it to digest the single-stranded loop portion of the double-stranded sequence and thereby form a split stem-loop oligonucleotide.

122. The method of any one of claims 118 to 121 wherein:

- the stem portion of the intact stem-loop oligonucleotide has a melting temperature (T_m) that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is below the T_m of the stem portion of the intact stem-loop oligonucleotide, and is above the T_m of the stem portion of the split stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

123. The method of any one of claims 118 to 122 wherein the T_m of the stem portion of the split stem-loop oligonucleotide is above the first temperature.

124. The method of any one of claims 118 to 123 wherein the T_m of the stem portion of the intact and split stem-loop oligonucleotide(s) is above the T_m of the first double-stranded portion of the multi-component temperature-controlled probe.

125. The method of any one of claims 57 to 116 wherein:

the second nucleic acid probe is a stem-loop oligonucleotide comprising a double-stranded stem portion of hybridised nucleotides opposing strands of which are linked by an unbroken single-stranded loop portion of unhybridized nucleotides of which all or a portion is complementary to the second target, and wherein

the modification of the second nucleic acid probe is a conformational change arising from hybridisation of the second target to the single-stranded loop portion by

complementary base pairing that causes spatial separation of the third and fourth detection moieties.

126. The method of claim 125 wherein the conformational change is dissociation of the opposing strands in the double-stranded stem portion of the second nucleic acid probe.

127. The method of any one of claims 118 to 126 wherein the third and fourth detection moieties are connected to opposing strands of the double-stranded stem portion of the second nucleic acid probe.

128. The method of any one of claims 57 to 116, wherein:

- the second target is a nucleic acid,
- the mixture further comprises:
 - a primer complementary to a first sequence in the second target,
 - a pitcher oligonucleotide comprising a region complementary to a second sequence in the second target that differs from the first sequence, and a tag portion that is not complementary to the second target,
 - a first polymerase comprising exonuclease activity, and
 - optionally a second polymerase, and
- said treating the mixture comprises:
 - suitable conditions to hybridise the primer and the pitcher oligonucleotide to the second target,
 - extending the primer using the first or second polymerase and the second target as a template to thereby cleave off the tag portion,
 - hybridising the cleaved tag portion to the second nucleic acid probe by complementary base pairing,
 - and extending the tag portion using the polymerase and the second nucleic acid probe as a template to generate a double-stranded catcher sequence comprising the second nucleic acid probe thereby providing said modification to the second nucleic acid probe and enabling the third and fourth detection moieties to provide the second detectable signal.

129. The method of claim 128 wherein:

- the double-stranded catcher sequence has a T_m that is above the first temperature; and
- the second temperature is below the T_m of the double-stranded catcher sequence.

130. The method of claim 128 or claim 129 wherein said extending the tag portion spatially separates the third and fourth detection moieties to thereby generate the second detectable signal.

131. The method of any one of claims 57 to 116 wherein the second target is a nucleic acid and the second nucleic acid probe is a two-part probe comprising a first part oligonucleotide and a second part oligonucleotide, wherein:

- the first part oligonucleotide is complementary to a first portion of the second target,
- the second part oligonucleotide is complementary to a second portion of the second target,
- the first and second portions of the second nucleic acid target flank one another but do not overlap,
- said treating the mixture comprises:
 - forming a duplex structure comprising:
 - a first double-stranded portion by hybridising the first part oligonucleotide to the second target by complementary base pairing, and
 - a second double-stranded portion by hybridising the second part oligonucleotide to the second target by complementary base pairing,
 - thereby bringing the first and second part oligonucleotides into proximity and providing said modification to the second nucleic acid probe enabling the third and fourth detection moieties to come into close proximity and generate the second detectable signal.

132. The method of claim 131 wherein the second detectable signal is a decrease in fluorescence.

133. The method of claim 131 wherein the second detectable signal is an increase in fluorescence.

134. The method of any one of claims 57 to 116, wherein:

- the second target is a nucleic acid,
- the second nucleic acid probe comprises a sequence that is complementary to the second target,
- the mixture further comprises:
 - a primer complementary to a portion of the second target, and
 - a polymerase with exonuclease activity;
- said treating the mixture comprises:
 - hybridising the primer to the second target by complementary base pairing,
 - hybridising the second nucleic acid probe to the second target by complementary base pairing,
 - extending the primer using the polymerase and the second target as a template to thereby digest the second nucleic acid probe and provide said modification to the second nucleic acid probe enabling the third and fourth detection moieties to spatially separate and generate the second detectable signal.

135. The method of any one of claims 57 to 116, wherein:

- the second target is a nucleic acid,
- the mixture further comprises a restriction endonuclease capable of digesting a double-stranded duplex comprising the second target; and
- said treating the mixture comprises:
 - hybridising the second nucleic acid probe to the second target by complementary base pairing to thereby form the double-stranded duplex,
 - digesting the duplex using the restriction endonuclease to thereby provide said modification to the second nucleic acid probe and enabling the third and fourth detection moieties to spatially separate and generate the second detectable signal.

136. The method of any one of claims 57 to 116 wherein:

- the second nucleic acid probe is a second multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,
 - wherein the first oligonucleotide component of the second multi-component temperature-controlled probe comprises a capture region capable of hybridisation to the second oligonucleotide component of the second multi-

component temperature-controlled probe by complementary base pairing to form a double-stranded portion,

wherein the first oligonucleotide component of the second multi-component temperature-controlled probe further comprises a sensor region capable of serving as a substrate for a second enzyme only when the second target is present in the sample,

wherein the first oligonucleotide component of the second multi-component temperature-controlled probe is connected to the third detection moiety and the second oligonucleotide component of the second multi-component temperature-controlled probe is connected to the fourth detection moiety,

- the mixture further comprises the second enzyme,

- said treating of the mixture comprises:

suitable conditions for the second enzyme to digest the sensor region of the second multi-component temperature-controlled probe to thereby generate a first fragment comprising the capture region and a second fragment connected to the third detection moiety, thereby enabling the third and fourth detection moieties to spatially separate and generate a second detectable signal.

137. The method of claim 136 wherein the first enzyme is the same as the second enzyme.

138. The method of claim 136 or claim 137 wherein the double-stranded portion or portions of the second multi-component temperature-controlled probe has a T_m above the T_m of the first double stranded portion and/or the second double stranded portion of the first multi-component temperature-controlled probe.

139. The method of any one of claims 57 to 138 wherein:

- the first target is a nucleic acid;
- the second target is a nucleic acid; or
- the first target is a nucleic acid and the second target is a nucleic acid.

140. The method of claim 139 wherein the first target and/or the second target is an amplicon of a nucleic acid.

141. The method of claim 140 wherein the amplicon is produced by an amplification reaction selected from the group consisting of polymerase chain reaction (PCR), strand displacement amplification (SDA), nicking enzyme amplification reaction (NEAR), helicase dependent amplification (HDA), Recombinase Polymerase Amplification (RPA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), transcription-mediated amplification (TMA), self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), Ligase Chain Reaction (LCR) or Ramification Amplification Method (RAM) and reverse transcription polymerase chain reaction (RT-PCR).

142. The method of claim 141 wherein said determining:

- occurs prior to said amplification or within 1, 2, 3, 4, or 5 cycles of said amplification commencing; and/or
- occurs after completion of said amplification.

143. The method of any one of claims 140 to 142 wherein said determining the presence or absence of the first and second targets comprises a melt curve analysis.

144. The method of any one of claims 57 to 116 or 136 to 143 wherein:

- the mixture further comprises a DNAzyme or a ribozyme requiring a co-factor for catalytic activity;
- said treating of the mixture comprises using conditions suitable for:
 - binding of the co-factor to the DNAzyme or ribozyme to render it catalytically active,
 - hybridisation of the DNAzyme or ribozyme to the second nucleic acid probe by complementary base pairing, and
 - catalytic activity of the DNAzyme or ribozyme to thereby digest the second nucleic acid probe and thereby provide said modification to the second nucleic acid probe enabling the third and fourth detection moieties to spatially separate and generate the second detectable signal, and
- the second target is the co-factor.

145. The method of any one of claims 57 to 106 or 117 to 144 wherein:

- the first enzyme is a DNAzyme or a ribozyme requiring a co-factor for catalytic activity,
 - said treating of the mixture comprises using conditions suitable for:
 - binding of the cofactor to the DNAzyme or ribozyme to render it catalytically active,
 - hybridisation of the DNAzyme or ribozyme to the first oligonucleotide component by complementary base pairing,
 - catalytic activity of the DNAzyme or ribozyme to thereby digest the first oligonucleotide component and generate the first fragment and the second fragment, and
 - the first target is the co-factor.

146. The method of claim 144 or claim 145 wherein the co-factor is a metal ion, such as a metal ion selected from: Mg^{2+} , Mn^{2+} , Ca^{2+} and Pb^{2+} .

147. The method of any one of claims 57 to 106 or 117 to 144 wherein the first enzyme is an aptazyme wherein:

- the sensor region comprises a substrate for an aptazyme;
- the first target is an analyte, protein, peptide, compound or nucleic acid;
- the mixture comprises an aptazyme comprising an aptamer capable of binding to the first target; and
- said treating the mixture further comprises binding of the aptazyme to the first target and to the sensor region to facilitate cleavage of the first oligonucleotide component to thereby generate the first fragment and the second fragment.

148. The method of any one of claims 57 to 147 wherein generation of the first detectable signal is not reversible at the first temperature.

149. A method for determining the presence or absence of a first target and a second target in a sample, the method comprising:

- (a) preparing a mixture for a reaction by contacting the sample or a derivative thereof putatively comprising the first and/or second target with:

- a multi-component temperature-controlled probe for detection of the first target, the multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,

wherein the first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a first double-stranded portion,

wherein the first oligonucleotide component further comprises a sensor region capable of serving as a substrate for an enzyme,

wherein the first oligonucleotide component is connected to a first detection moiety and the second oligonucleotide component is connected to a second detection moiety,

- a second nucleic acid probe for detection of the second target, the second nucleic acid probe comprising third and fourth detection moieties,

wherein the first and second detection moieties are capable of generating a first detectable signal, and the third and fourth detection moieties are capable of generating a second detectable signal, and

- a first enzyme capable of modifying the sensor region of the first oligonucleotide component only when the first target is present in the sample;

(b) treating the mixture under conditions suitable for:

- the first enzyme to modify the sensor region of the first oligonucleotide component to thereby generate a first fragment comprising the first capture region and a second fragment connected to the first detection moiety, thereby enabling the first and second detection moieties to spatially separate and generate a first detectable signal,

- the second target to induce a modification of the second nucleic acid probe, thereby enabling the third and fourth detection moieties to spatially separate and generate a second detectable signal;

(c) measuring a level of background or detectable signal:

- at a first temperature at or below which the first capture region is hybridised to the second oligonucleotide component,

- at a second temperature at or below which the first capture region is hybridised to the second oligonucleotide component,

(d) determining whether at one or more timepoints during or after said treating:

- a first detectable signal is generated at the second temperature at or below which the first capture region is hybridised to the second oligonucleotide component,

- a second detectable signal arising from said modification of the second nucleic acid probe is generated at the first temperature, wherein the second detectable signal is indicative of the presence of the second target in the sample.

150. The method of any one of claims 57 to 149 wherein the first temperature is lower than the second temperature.

151. The method of any one of claims 57 to 150 wherein the first enzyme does not digest the first target and/or the second target.

152. The method of any one of claims 57 to 151 wherein the first temperature differs from the second temperature by more than: 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C or 60°C.

153. The method of any one of claims 57 to 152 wherein the biological sample is obtained from a subject.

154. The method of any one of claims 57 to 153 wherein the method is performed *in vitro*.

155. The method of any one of claims 57 to 153 wherein the method is performed *ex vivo*.

156. A composition comprising:

a multi-component temperature-controlled probe comprising a first oligonucleotide component and a second oligonucleotide component,

wherein the first oligonucleotide component comprises a first capture region capable of hybridisation to the second oligonucleotide component by complementary base pairing to form a first double-stranded portion,

wherein the first oligonucleotide component is connected to a first detection moiety and the second oligonucleotide component is connected to a second detection moiety,

wherein:

the first oligonucleotide component further comprises a sensor region capable of serving as a substrate for an enzyme,
wherein digestion of the sensor region by the enzyme generates a first fragment and a second fragment,
and wherein the first fragment comprises the first capture region,
and the second fragment is connected to the first detection moiety;
and wherein:
the multi-component temperature-controlled probe does not comprise more than two detection moieties;
the first oligonucleotide component is not connected to more than one detection moiety; or
the first fragment is not directly labelled with a detection moiety.

157. The composition of claim 156 wherein the first oligonucleotide component is hybridised to the second oligonucleotide component by complementary base pairing at the first capture region.

158. The composition of claim 156 or claim 157 wherein the second oligonucleotide component is not directly labelled with more than one detection moiety.

159. The composition of any one of claims 156 to 158 wherein the second oligonucleotide component is not connected to more than one detection moiety.

160. The composition of any one of claims 156 to 159 wherein the second oligonucleotide component is directly labelled with the second detection moiety.

161. The composition of any one of claims 156 to 160 wherein the second fragment is not directly labelled with the first detection moiety.

162. The composition of any one of claims 156 to 161 wherein the first oligonucleotide component is not directly labelled with the first detection moiety.

163. The composition of claim 162 wherein the first oligonucleotide component is connected to the first detection moiety via a third oligonucleotide component, wherein

the first oligonucleotide component further comprises a second capture region capable of hybridisation to the third oligonucleotide component by complementary base pairing to form a second double-stranded portion, and

the third oligonucleotide component is directly labelled with the first detection moiety.

164. The composition of claim 163 wherein the first capture region differs in length or sequence from the second capture region.

165. The composition of any one of claims 156 to 160 wherein the first oligonucleotide component is directly labelled with the first detection moiety.

166. The composition of any one of claims 156 to 165 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore.

167. The composition of any one of claims 156 to 166 wherein:

the first detection moiety is a fluorophore, and the second detection moiety is a quencher; or

the first detection moiety is a quencher, and the second detection moiety is a fluorophore;

and wherein the multi-component temperature-controlled probe does not comprise more than one quencher.

168. The composition of any one of claims 156 to 167 wherein the sensor region is located between the first capture region and the first detection moiety.

169. The composition of any one of claims 156 to 168 wherein following digestion of the sensor region the first fragment is capable of hybridizing to the second oligonucleotide component via the first capture region.

170. The composition of any one of claims 156 to 169, further comprising a multi-component nucleic acid enzyme (MNAzyme) comprising two partzyme oligonucleotides, each partzyme oligonucleotide having a substrate arm capable of hybridising to at least a portion of the sensor region of the first oligonucleotide component.

171. The composition of claim 170 wherein the substrate arms of the two partzyme oligonucleotides are hybridised to the sensor region of the first oligonucleotide component.

172. The composition of any one of claims 156 to 171, further comprising a DNAzyme capable of cleaving the sensor region of the first oligonucleotide component only in the presence of a target.

173. The composition of any one of claims 156 to 172, further comprising an aptazyme capable of cleaving the sensor region of the first oligonucleotide component only in the presence of a target.

174. The composition of any one of claims 156 to 173, further comprising a restriction endonuclease capable of cleaving the sensor region of the first oligonucleotide component only in the presence of a nucleic acid target.

175. The composition of any one of claims 156 to 174, further comprising an exonuclease capable of digesting the sensor region of the first oligonucleotide component only in the presence of a nucleic acid target.

176. The composition of claim 175 wherein the exonuclease is a polymerase with exonuclease activity.