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 Tm 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 of 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

Provided herein are methods and compositions which extend the capacity for multiplex analysis of targets.

In a first aspect, the present disclosure provides a method for determining the presence or absence of a first 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;

- 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,

(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 first 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 first target in the sample.

In a second aspect, the present disclosure provides 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.

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 (Tm) of the double-stranded portion (Tm 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 Tm of the complementary regions (Tm OC1/OC3). If this temperature is greater than the Tm OC1/OC2, then at temperatures below Tm 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 Tm OC1/OC2. At temperatures above the Tm 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 Tm OC1/OC2 but no change in fluorescence will be observed, regardless of the presence or absence of target, at temperatures above the Tm 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 MNAzyme). 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 examples, 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 Tm OC1/OC2, an increase in fluorescence is indicative of the presence of the target, which facilitated assembly of the PlexZyme. At temperatures above the Tm 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 examples, 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 examples 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 Tm OC1/OC2, an increase in fluorescence is indicative of the presence of the target. At temperatures above the Tm OC1/OC2, no change in fluorescence will be observed regardless of the presence or absence of the target. In some examples 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 examples 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 Tm OC1/OC2, an increase in fluorescence is indicative of the presence of the target. At temperatures above the Tm 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 examples, 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 examples, 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 examples, 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 examples, M-Tec Probes are used in combination with LOCS Probes. In other examples, 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 examples 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 examples:

Example 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.

Example 2. The method of Example 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.

Example 3. The method of Example 1 or Example 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.

Example 4. The method of Example 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.

Example 5. The method of Example 3 or Example 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.

Example 6. The method of Example 1 or Example 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.

Example 7. The method of Example 1 or Example 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.

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

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

Example 10. The method of Example 1 or Example 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.

Example 11. The method of any one of Examples 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.

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

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

Example 14. The method of Example 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.

Example 15. The method of Example 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.

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

Example 17. The method of Example 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.

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

Example 19. The method of any one of Examples 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.

Example 20. The method of any one of Examples 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.

Example 21. The method of Example 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.

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

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

Example 24. The method of Example 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).

Example 25. The method of Example 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.

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

Example 27. The method of any one of Examples 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.

Example 28. The method of Example 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+}.

Example 29. The method of any one of Examples 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.

Example 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.

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

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

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

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

Example 35. The method of any one of Examples 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.

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

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

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

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

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

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

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

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

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

Example 45. The method of any one of Examples 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.

Example 46. The method of any one of Examples 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.

Example 47. The method of any one of Examples 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.

Example 48. The method of any one of Examples 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.

Example 49. The method of any one of Examples 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.

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

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

Example 52. The method of any one of Examples 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.

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

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

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

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

Example 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.

Example 58. The method of Example 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.

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

Example 60. The method of Example 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.

Example 61. The method of any one of Examples 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.

Example 62. The method of Example 57 or Example 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.

Example 63. The method of Example 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.

Example 64. The method of any one of Examples 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.

Example 65. The method of any one of Examples 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.

Example 66. The method of any one of Examples 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.

Example 67. The method of any one of Examples 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.

Example 68. The method of any one of Examples 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.

Example 69. The method of Example 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.

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

Example 71. The method of any one of Examples 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.

Example 72. The method of any one of Examples 57 to 67 wherein part (c) comprises measuring a first background signal at or within $1^{o}C$, $2^{o}C$, $3^{o}C$, $4^{o}C$ or $5^{o}C$ 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.

Example 73. The method of Example 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.

Example 74. The method of any one of Examples 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.

Example 75. The method of any one of Examples 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.

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

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

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

Example 79. The method of any one of Examples 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.

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

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

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

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

Example 84. The method of any one of Examples 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.

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

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

Example 87. The method of any one of Examples 84 to 86 wherein the Tm of the first double-stranded portion is less than the Tm of the second double-stranded portion.

Example 88. The method of any one of Examples 84 to 86 wherein the Tm of the second double-stranded portion is less than the Tm of the first double-stranded portion.

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

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

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

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

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

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

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

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

Example 97. The method of any one of Examples 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.

Example 98. The method of any one of Examples 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.

Example 99. The method of any one of Example 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.

Example 100. The method of any one of Examples 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.

Example 101. The method of any one of Examples 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.

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

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

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

Example 105. The method of any one of Examples 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.

Example 106. The method of any one of Examples 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.

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

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

Example 109. The method of Example 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.

Example 110. The method of Example 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.

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

Example 112. The method of Example 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.

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

Example 114. The method of any one of Examples 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.

Example 115. The method of any one of Examples 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.

Example 116. The method of Example 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.

Example 117. The method of any one of Examples 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.

Example 118. The method of Example 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.

Example 119. The method of Example 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.

Example 120. The method of any one of Examples 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.

Example 121. The method of any one of Examples 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.

Example 122. The method of any one of Examples 118 to 121 wherein:

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

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

- the first temperature is below the second temperature.

Example 123. The method of any one of Examples 118 to 122 wherein the Tm of the stem portion of the split stem-loop oligonucleotide is above the first temperature.

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

Example 125. The method of any one of Examples 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.

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

Example 127. The method of any one of Examples 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.

Example 128. The method of any one of Examples 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.

Example 129. The method of Example 128 wherein:

- the double-stranded catcher sequence has a Tm that is above the first temperature; and

- the second temperature is below the Tm of the double-stranded catcher sequence.

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

Example 131. The method of any one of Examples 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.

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

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

Example 134. The method of any one of Examples 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.

Example 135. The method of any one of Examples 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.

Example 136. The method of any one of Examples 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.

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

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

Example 139. The method of any one of Examples 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.

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

Example 141. The method of Example 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).

Example 142. The method of Example 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.

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

Example 144. The method of any one of Examples 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.

Example 145. The method of any one of Examples 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.

Example 146. The method of Example 144 or Example 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+}.

Example 147. The method of any one of Examples 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.

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

Example 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.

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

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

Example 152. The method of any one of Examples 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.

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

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

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

Example 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.

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

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

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

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

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

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

Example 163. The composition of Example 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.

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

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

Example 166. The composition of any one of Examples 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.

Example 167. The composition of any one of Examples 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.

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

Example 169. The composition of any one of Examples 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.

Example 170. The composition of any one of Examples 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.

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

Example 172. The composition of any one of Examples 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.

Example 173. The composition of any one of Examples 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.

Example 174. The composition of any one of Examples 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.

Example 175. The composition of any one of Examples 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.

Example 176. The composition of Example 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