curve. This process is often slow and typically takes anywhere between 30-60 mins to complete. Furthermore, melt curve analyses can require interpretation by skilled personel and/or use of specialised software for results interpretation. Hence, there is a high demand for faster and/or simpler alternatives to melt curve analyses.

A need exists for improved compositions and methods for thesimultaneous detection, differentiation, and/or quantification of multiple unrelated amplicons generated by PCR or by alternative target amplification protocols.

Summary of the Invention

In one aspect, provided herein is a method for determining the presence or absence of first and second targets in a sample, the method comprising: (a) preparing a reaction mixture by contacting the sample or a derivative thereof putatively comprising the first and/or second targets or amplicons thereof with: first and second closed stem-loop oligonucleotides, wherein each of the closed stem-loop oligonucleotides comprise a double-stranded stem portion of hybridised nucleotides connected to a closed single-stranded loop portion of unhybridised nucleotides, wherein: the number of hybridised nucleotides and/or the sequence of hybridised nucleotides in the stem portion of the first and second closed stem-loop oligonucleotide differs, and enzymes capable of cleaving or degrading the single-stranded loop portion of the first and second closed stem-loop oligonucleotides only when in contact with the target or an amplicon thereof; (b) treating the reaction mixture: - under conditions suitable for the enzymes to induce cleavage or degradation of the loop portion of the first and second closed stem-loop oligonucleotides to thereby produce first and second open stem-loop oligonucleotides; - at a first temperature at or above which strands of the double-stranded stem portion of the first open stem-loop oligonucleotide disassociate to thereby facilitate spatial separation of the fluorophore and quencher molecules of the stem portion of the first open stem-loop oligonucleotide and provide a first detectable fluorescent signal, and - at a second temperature at or above which strands of the double-stranded stem portion of the second open stem-loop oligonucleotide disassociate to thereby facilitate spatial separation of the fluorophore and quencher molecules of the stem portion of the second open stem-loop oligonucleotide and provide a second detectable fluorescent signal; wherein: the first temperature is lower than the second temperature, and the fluorophore of the first open stem-loop oligonucleotide and the fluorophore of the second open stem-loop oligonucleotide emit in the same colour region of the visible spectrum; and (c) detecting levels of said first and second fluorescent signals at one or more temperatures comprising or consisting of a

temperature equal to or above the second temperature, to thereby determine the presence or absence of said targets in the sample.

In another aspect, provided herein is a composition comprising: first and second closed stem-loop oligonucleotides, wherein each of the closed stem-loop oligonucleotides comprise a double-stranded stem portion of hybridised nucleotides connected to a single-stranded loop portion of unhybridised nucleotides, wherein: the number of hybridised nucleotides and/or the sequence of hybridised nucleotides in the stem portion of the first and second closed stem-loop oligonucleotide differs, each of the double-stranded stem portions comprise a fluorophore molecule connected to one strand and a quencher molecule connected to an opposing strand, and the single-stranded loop portions comprise a region that is capable of serving as a substrate for a catalytic nucleic acid, an exonuclease or an endonuclease, wherein the fluorophore molecules emit in the same colour region of the visible spectrum, and the melting temperature (Tm) of the double-stranded stem portion of the first closed stem-loop oligonucleotide differs from the Tm of the double-stranded stem portion of the second closed stem-loop oligonucleotide.

The present invention addresses one or more deficiencies existing in current multiplex detection assays.

Provided herein are methods and compositions which extend the capacity to multiplex during amplification protocols by using oligonucleotide structures referred to herein as LOCS (Loops Connected to Stems). A series of LOCS, labelled with a single fluorophore and quencher pair can be individually discriminated within a single reaction by virtue of the temperature at which the stem portion melts following opening of the LOCS by cleavage or degradation in response to the presence of target. The melting temperature of the stem region thus acts as a surrogate marker for the specific target which opened the LOCS. While other methods incorporating stem-loop structures have exploited the change in fluorescence following either a) hybridization of the loop region to the target amplicons (Molecular Beacons & Sloppy Beacons) which increases the distance between dye pairs, or b) by cleavage allowing physical separation of the dyes (Cleavable Molecular Beacons), the present invention provides improvements over existing multiplex detection assays. These improvements arise, at least in part, through manipulating the melting temperature of the stem portion of stem-loop oligonucleotides by changing the length and/or sequence composition of the stem such that each stem melts at a different temperature.

As described herein, multiple LOCS labelled with the same fluorophore may contain a) different loop sequences which allow direct or indirect detection of multiple targets

simultaneously and b) different stem sequences that melt at discreet temperatures and which can be used to identify the specific target(s) present within the multiple targets under investigation. The methods of the present invention provide one or more advantages over art-known methods such as, for example, the TOCE protocol in that separate catcher molecules are not required thus reducing the number of components in the reaction mix and reducing costs. Furthermore, the TOCE method is inherently more complex than the methods of the

CLAIMS

1. A method for determining the presence or absence of first and second targets in a sample, the method comprising:

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

first and second closed stem-loop oligonucleotides, wherein each of the closed stem-loop oligonucleotides comprise a double-stranded stem portion of hybridised nucleotides connected to a closed single-stranded loop portion of unhybridised nucleotides, wherein:

the number of hybridised nucleotides and/or the sequence of hybridised nucleotides in the stem portion of the first and second closed stem-loop oligonucleotide differs, and

enzymes capable of cleaving or degrading the single-stranded loop portion of the first and second closed stem-loop oligonucleotides only when in contact with the target or an amplicon thereof;

(b) treating the reaction mixture:

- under conditions suitable for the enzymes to induce cleavage or degradation of the loop portion of the first and second closed stem-loop oligonucleotides to thereby produce first and second open stem-loop oligonucleotides;
- at a first temperature at or above which strands of the double-stranded stem portion of the first open stem-loop oligonucleotide disassociate to thereby facilitate spatial separation of the fluorophore and quencher molecules of the stem portion of the first open stem-loop oligonucleotide and provide a first detectable fluorescent signal, and
- at a second temperature at or above which strands of the double-stranded stem portion of the second open stem-loop oligonucleotide disassociate to thereby facilitate spatial separation of the fluorophore and quencher molecules of the stem portion of the second open stem-loop oligonucleotide and provide a second detectable fluorescent signal;

wherein:

the first temperature is lower than the second temperature, and

the fluorophore of the first open stem-loop oligonucleotide and the fluorophore of the second open stem-loop oligonucleotide emit in the same colour region of the visible spectrum; and

(c) detecting levels of said first and second fluorescent signals at one or more temperatures comprising or consisting of a temperature equal to or above the second temperature, to thereby determine the presence or absence of said targets in the sample.

2. The method of claim 1, wherein the enzymes comprise multi-component nucleic acid enzymes (MNAzymes), and said treating of the reaction mixture comprises treating the reaction mixture under conditions suitable for:

binding of a first multi-component nucleic acid enzyme (MNAzyme) to the first target or amplicon thereof and hybridisation of substrate arms of said first MNAzyme to the loop portion of the first closed stem-loop oligonucleotide, to thereby facilitate said cleavage of the loop portion of the first closed stem-loop oligonucleotide by the first MNAzyme forming the first open stem-loop oligonucleotide.

3. The method of claim 2, wherein the first target is a nucleic acid sequence or amplicon thereof capable of hybridising to the sensor arms of the first MNAzyme to thereby facilitate assembly of the first MNAzyme.

4. The method of claim 1, wherein:

- the first target is an analyte, protein, compound or molecule;
- the enzymes comprise enzymes with an aptamer capable of binding to the first target; and
- binding of the first target to the aptamer is capable of rendering the enzymes with an aptamer catalytically active.

5. The method of claim 4, wherein the enzymes with an aptamer comprise any one or more of: apta-DNAzymes, apta-ribozymes, apta-MNAzymes.

6. The method of any one of claims 2, 4, or 5, wherein:

- the first target is an analyte, protein, compound or molecule;
- the reaction mixture further comprises an oligonucleotide sequence capable of hybridising to the sensor arms of the first MNAzyme to thereby facilitate assembly of the first MNAzyme;
- the first MNAzyme comprises an aptamer sequence capable of binding to the first target; and
- binding of the target to the aptamer is capable of rendering the first MNAzyme catalytically active.

7. The method of any one of claims 1 to 6, wherein said closed stem-loop oligonucleotides are not hybridised to said target or amplicon thereof during said cleavage or degradation by the enzymes.

8. The method of any one of claims 1 to 6, wherein the enzymes comprise restriction endonucleases, and said treating of the reaction mixture comprises:

treating the reaction mixture under conditions suitable for hybridisation of a first target or amplicon thereof to the loop portion of the first closed stem-loop oligonucleotide to form a double-stranded sequence for a first restriction endonuclease to associate with and thereby facilitate said cleavage of the loop portion of the first closed stem-loop oligonucleotide forming the first open stem-loop oligonucleotide.

9. The method of claim 8, wherein the restriction endonuclease is a nicking endonuclease capable of associating with and cleaving a loop strand of said double-stranded sequence for the first restriction endonuclease.

10. The method of any one of claims 1 to 6, wherein the enzymes comprise exonuclease activity (e.g. polymerase enzymes, exonucleases), and said treating of the reaction mixture comprises:

treating the reaction mixture under conditions suitable for:

- hybridisation of a first target or amplicon thereof to the loop portion of the first closed stem-loop oligonucleotide to form a first double-stranded sequence comprising the first target or amplicon thereof,
- hybridisation of a first primer oligonucleotide to the first target or amplicon thereof to form a second double-stranded sequence located upstream (5') relative to the first double-stranded sequence comprising the first target or amplicon thereof
- association of a first enzyme comprising exonuclease activity with a loop portion of the first closed stem-loop oligonucleotide at or adjacent to a terminus of the first primer oligonucleotide, and
- catalytic activity of the first enzyme comprising exonuclease activity to thereby facilitate degradation of the loop portion of the first double-stranded sequence comprising the first target or amplicon and form the first open stem-loop oligonucleotide.

11. The method of any one of claims 1 to 6, wherein the enzymes comprise exonuclease activity, and said treating of the reaction mixture comprises:

treating the reaction mixture under conditions suitable for:

- hybridisation of a first target or amplicon thereof to the loop portion of the first closed stem-loop oligonucleotide to form a first double-stranded sequence comprising the first target or amplicon thereof,
- association of a first enzyme comprising exonuclease activity with the first double-stranded sequence comprising the first target or amplicon thereof, and
- catalytic activity of the first enzyme comprising exonuclease activity to thereby facilitate degradation of the loop portion of the first double-stranded sequence comprising the first target or amplicon and form the first open stem-loop oligonucleotide.

12. The method of any one of claims 1 to 11, wherein the enzymes comprise DNAzymes and/or ribozymes requiring a first co-factor for catalytic activity, and said treating of the reaction mixture comprises treating the reaction mixture under conditions suitable for:

- binding of a said first cofactor to the DNAzyme and/or binding of a said first cofactor to the ribozyme to render the DNAzyme and/or ribozyme catalytically active,
- hybridisation of DNAzyme and/or ribozyme to the loop portion of a first closed stem-loop oligonucleotide,
- catalytic activity of the DNAzyme and/or ribozyme to thereby facilitate cleavage of the loop portion of the first closed stem-loop oligonucleotide and form the first open stem-loop oligonucleotide.

wherein:

the first target is the first co-factor.

13. The method of claim 12, wherein the first co-factor is a metal ion (e.g. Mg^{2+}, Mn^{2+}, Ca^{2+}, Pb^{2+}).

14. The method of any one of claims 1 to 13, wherein said treating further comprises treating the reaction mixture under conditions suitable for any one or more of:

- binding of a second MNAzyme to the second target or amplicon thereof and hybridisation of substrate arms of said second MNAzyme to the loop portion of the second closed stem-loop oligonucleotide, to thereby facilitate said cleavage of the loop portion of the second closed stem-loop oligonucleotide by the second MNAzyme forming the second open stem-loop oligonucleotide;

- hybridisation of a second target or amplicon thereof to the loop portion of the second closed stem-loop oligonucleotide to form a double-stranded sequence for a second restriction endonuclease to associate with the double-stranded sequence and thereby facilitate said cleavage of the loop portion of the second closed stem-loop oligonucleotide forming the second open stem-loop oligonucleotide;
- hybridisation of a second target or amplicon thereof to the loop portion of the second closed stem-loop oligonucleotide to form a second double-stranded sequence comprising the second target or amplicon thereof,

hybridisation of a second primer oligonucleotide to the second target or amplicon thereof to form a second double-stranded sequence located upstream (5') relative to the second double-stranded sequence comprising the second target or amplicon thereof,

association of a second enzyme comprising exonuclease activity with a loop portion of the second closed stem-loop oligonucleotide at or adjacent to a terminus of the second primer oligonucleotide, and

catalytic activity of the second enzyme comprising exonuclease activity to thereby facilitate degradation of the loop portion of the second double-stranded sequence comprising the second target or amplicon and form the second open stem-loop oligonucleotide;

- hybridisation of a second target or amplicon thereof to the loop portion of the second closed stem-loop oligonucleotide to form a second double-stranded sequence comprising the second target or amplicon thereof,

association of a second enzyme comprising exonuclease activity with the second double-stranded sequence comprising the second target or amplicon thereof, and

catalytic activity of the second enzyme comprising exonuclease activity to thereby facilitate degradation of the loop portion of the second double-stranded sequence comprising the second target or amplicon and form the second open stem-loop oligonucleotide;

- binding of a second cofactor to a DNAzyme and/or binding of a second cofactor to a ribozyme to render the DNAzyme and/or ribozyme catalytically active,

hybridisation of DNAzyme and/or ribozyme to the loop portion of a second closed stem-loop oligonucleotide,

catalytic activity of the DNAzyme and/or ribozyme to thereby facilitate cleavage of the loop portion of the second closed stem-loop oligonucleotide and form the second open stem-loop oligonucleotide.

wherein:

the second target is the second co-factor.

15. The method of claim 14, wherein the second co-factor is a metal ion (e.g. Mg^{2+}, Mn^{2+}, Ca^{2+}, Pb^{2+}).

16. The method of claim 14, wherein the second restriction endonuclease is a nicking endonuclease capable of associating with and cleaving a loop strand of said double-stranded sequence comprising the second target or amplicon thereof.

17. The method of claim 16, wherein the first and second restriction endonucleases are a different type of restriction endonuclease.

18. The method of claim 16, wherein the first and second restriction endonucleases are the same type of restriction endonuclease.

19. The method of claim 14, wherein the second target is a nucleic acid sequence or amplicon thereof capable of hybridising to the sensor arms of the second MNAzyme to thereby facilitate assembly of the second MNAzyme.

20. The method of claim 14, wherein:

- the second target is an analyte, protein, compound or molecule;
- the enzymes comprise enzymes with an aptamer capable of binding to the second target; and
- binding of the second target to the aptamer is capable of rendering the enzymes with an aptamer catalytically active.

21. The method of claim 20, wherein the enzymes with an aptamer comprise any one or more of: apta-DNAzymes, apta-ribozymes, apta-MNAzymes.

22. The method of claim 14, 20 or 21, wherein:

- the second target is an analyte, protein, compound or molecule;
- the reaction mixture further comprises an oligonucleotide sequence capable of hybridising to the sensor arms of the second MNAzyme to thereby facilitate assembly of the second MNAzyme;
- the second MNAzyme comprises an aptamer sequence capable of binding to the second target; and

- binding of the second target to the aptamer sequence of the second MNAzyme is capable of rendering the second MNAzyme catalytically active by facilitating removal of an inhibitory molecule bound to the aptamer of the second MNAzyme.

23. The method of any one of claims 1 to 22 wherein the fluorophore of the first closed and open stem-loop oligonucleotides is the same as the fluorophore of the second closed and open stem-loop oligonucleotides.

24. The method of any one of claims 14 to 23, wherein:

- the reaction mixture comprises said first and second MNAzymes; and
- the sequence of the loop portion of the first closed stem-loop oligonucleotide capable of hybridising to the substrate arms of the first MNAzyme is different from the sequence of the loop portion of the second closed stem-loop oligonucleotide capable of hybridising to the substrate arms of the second MNAzyme.

25. The method of any one of claims 1 to 24, wherein the fluorophore of the first closed and open stem-loop oligonucleotides and the fluorophore of the second closed and open stem-loop oligonucleotides are detectable in a single fluorescence emission channel of a device.

26. The method of any one of claims 1 to 25, wherein said enzymes do not induce cleavage or degradation of any said target or amplicon thereof.

27. The method of any one of claims 1 to 26, wherein the first and second closed stem-loop oligonucleotides each consist of said double-stranded stem portion and said single-stranded loop portion.

28. The method of any one of claims 1 to 27, further comprising determining the presence or absence of a third target or amplicon thereof in a sample by:

(d) contacting the reaction mixture comprising the sample or a derivative thereof with:

a third closed stem-loop oligonucleotide, wherein the third closed stem-loop oligonucleotide comprises a double-stranded stem portion of hybridised nucleotides connected to a closed single-stranded loop portion of unhybridised nucleotides, wherein:

the number of hybridised nucleotides and/or the sequence of hybridised nucleotides in the stem portion of the third closed stem-loop oligonucleotide differs from that of the stem portions of the first and second closed stem-loop oligonucleotide;

the double-stranded stem portion comprises a fluorophore molecule connected to one strand and a quencher molecule connected to an opposing strand; and

enzymes capable of cleaving or degrading the single-stranded loop portion of the third closed stem-loop oligonucleotide only when in contact with the target or amplicon thereof;

(e) treating the reaction mixture:

- under conditions suitable for the enzymes to induce cleavage or degradation of the loop portion of the third closed stem-loop oligonucleotide to thereby produce a third open stem-loop oligonucleotide;

- at a third temperature at or above which strands of the double-stranded stem portion of the third open stem-loop oligonucleotide disassociate to thereby facilitate spatial separation of the fluorophore and quencher molecules of the stem portion of the third open stem-loop oligonucleotide and provide a third detectable fluorescent signal;

wherein:

the third temperature is higher than the first and second temperatures, and

(f) detecting levels of said first, second and third fluorescent signals at one or more temperatures comprising or consisting of a temperature equal to or above the third temperature, to thereby determine the presence or absence of said targets in the sample.

29. The method of any one of embodiments 1 to 27, further comprising determining the presence or absence of a third target or amplicon thereof in a sample by:

(d) contacting the reaction mixture comprising the sample or a derivative thereof with:

a third closed stem-loop oligonucleotide, wherein the third closed stem-loop oligonucleotide comprises a double-stranded stem portion of hybridised nucleotides connected to a closed single-stranded loop portion of unhybridised nucleotides, wherein:

the number of hybridised nucleotides and/or the sequence of hybridised nucleotides in the stem portion of the third closed stem-loop oligonucleotide is the same as that of the stem portions of the first or second closed stem-loop oligonucleotide;

the double-stranded stem portion comprises a fluorophore molecule connected to one strand and a quencher molecule connected to an opposing strand, wherein the fluorophore molecule connected to the third open stem-loop oligonucleotide emits in a

different colour region of the visible spectrum than the fluorophore of the first and/or second closed stem-loop oligonucleotides; and

enzymes capable of cleaving or degrading the single-stranded loop portion of the third closed stem-loop oligonucleotide only when in contact with the target or amplicon thereof;

(e) treating the reaction mixture:

- under conditions suitable for the enzymes to induce cleavage or degradation of the loop portion of the third closed stem-loop oligonucleotide to thereby produce a third open stem-loop oligonucleotide;

- at a third temperature at which strands of the double-stranded stem portion of the third open stem-loop oligonucleotide disassociate to thereby facilitate spatial separation of the fluorophore and quencher molecules of the stem portion of the third open stem-loop oligonucleotide and provide a third detectable fluorescent signal

(f) detecting levels of said first, second and third fluorescent signals at one or more temperatures comprising or consisting of a temperature equal to or above the third temperature, to thereby determine the presence or absence of said targets in the sample.

30. The method of claim 29, wherein the third temperature differs from the first and/or second temperatures.

31. The method of any one of claims 28 to 30, wherein the method comprises treating the reaction mixture under conditions suitable for any one or more of:

- binding of a third MNAzyme to the third target or amplicon thereof and hybridisation of substrate arms of said third MNAzyme to the loop portion of the third closed stem-loop oligonucleotide, to thereby facilitate said cleavage of the loop portion of the third closed stem-loop oligonucleotide by the third MNAzyme forming the third open stem-loop oligonucleotide;

- hybridisation of the third target or amplicon thereof to the loop portion of the third closed stem-loop oligonucleotide to form a double-stranded sequence for a third restriction endonuclease to associate with the double-stranded sequence and thereby facilitate said cleavage of the loop portion of the third closed stem-loop oligonucleotide forming the third open stem-loop oligonucleotide;

- hybridisation of a third target or amplicon thereof to the loop portion of the third closed stem-loop oligonucleotide to form a third double-stranded sequence comprising the third target or amplicon thereof,

hybridisation of a third primer oligonucleotide to the third target or amplicon thereof to form a third double-stranded sequence located upstream (5') relative to the third double-stranded sequence comprising the third target or amplicon thereof, association of a third enzyme comprising exonuclease activity with a loop portion of the third closed stem-loop oligonucleotide at or adjacent to a terminus of the third primer oligonucleotide, and

catalytic activity of the third enzyme comprising exonuclease activity to thereby facilitate degradation of the loop portion of the third double-stranded sequence comprising the third target or amplicon and form the third open stem-loop oligonucleotide;

- hybridisation of a third target or amplicon thereof to the loop portion of the third closed stem-loop oligonucleotide to form a third double-stranded sequence comprising the third target or amplicon thereof,

association of a third enzyme comprising exonuclease activity with the third double-stranded sequence comprising the third target or amplicon thereof, and

catalytic activity of the third enzyme comprising exonuclease activity to thereby facilitate degradation of the loop portion of the third double-stranded sequence comprising the third target or amplicon and form the third open stem-loop oligonucleotide;

- binding of a third cofactor to a DNAzyme and/or binding of a third cofactor to a ribozyme to render the DNAzyme and/or ribozyme catalytically active,

hybridisation of DNAzyme and/or ribozyme to the loop portion of a third closed stem-loop oligonucleotide,

catalytic activity of the DNAzyme and/or ribozyme to thereby facilitate cleavage of the loop portion of the third closed stem-loop oligonucleotide and form the third open stem-loop oligonucleotide.

wherein:

the third target is the third co-factor.

32. The method of claim 31, wherein the co-factor is a metal ion (e.g. Mg^{2+}, Mn^{2+}, Ca^{2+}, Pb^{2+}).

33. The method of claim 31, wherein the restriction endonuclease is a nicking endonuclease capable of associating with and cleaving a loop strand of said double-stranded sequence comprising the third target or amplicon thereof.

34. The method of claim 31, wherein the third target is a nucleic acid sequence or amplicon thereof capable of hybridising to the sensor arms of the third MNAzyme to thereby facilitate assembly of the third MNAzyme.

35. The method of claim 31, wherein:

- the target is an analyte, protein, compound or molecule;
- the enzymes comprise enzymes with an aptamer capable of binding to the third target; and
- binding of the third target to the aptamer is capable of rendering the enzymes with an aptamer catalytically active

36. The method of claim 35, wherein the enzymes with an aptamer comprise any one or more of: apta-DNAzymes, apta-ribozymes, apta-MNAzymes.

37. The method of claim 31, wherein:

- the third target is an analyte, protein, compound or molecule;
- the reaction mixture further comprises an oligonucleotide sequence capable of hybridising to the sensor arms of the third MNAzyme to thereby facilitate assembly of the third MNAzyme;
- the third MNAzyme comprises an aptamer sequence capable of binding to the third target; and
- binding of the third target to the aptamer sequence of the third MNAzyme is capable of rendering the third MNAzyme catalytically active by facilitating removal of an inhibitory molecule bound to the aptamer of the third MNAzyme.

38. The method of any one of claims 31, or 33 to 37, wherein:

- the reaction mixture comprises said third MNAzyme and either of both of said first and second MNAzymes; and
- the sequence of the loop portion of the third closed stem-loop oligonucleotide capable of hybridising to the substrate arms of the third MNAzyme is different from:

  the sequence of the loop portion of the first closed stem-loop oligonucleotide capable of hybridising to the substrate arms of the first MNAzyme, and/or

the sequence of the loop portion of the second closed stem-loop oligonucleotide capable of hybridising to the substrate arms of the first MNAzyme.

39. The method of any one of claims 28 or claims 31 to 38, wherein:

- the number of hybridised nucleotides and/or the sequence of hybridised nucleotides in the stem portion of the third closed stem-loop oligonucleotide differs from that of the stem portions of the first and second closed stem-loop oligonucleotides;
- the third temperature differs from the first and second temperatures; and
- the fluorophore of the third closed and open stem-loop oligonucleotides emits in the same colour region of the visible spectrum as the fluorophores of the first and second closed and open stem-loop oligonucleotides.

40. The method of any one of claims 28 or claims 31 to 39, wherein the fluorophore of the third closed and open stem-loop oligonucleotides is the same as the fluorophore of the first and/or second closed and open stem-loop oligonucleotides.

41. The method of claim 40, wherein the fluorophore of the third closed and open stem-loop oligonucleotides and the fluorophore of the first closed and open stem-loop oligonucleotides and/or the fluorophore of the second closed and open stem-loop oligonucleotides are detectable using the same fluorescence emission channel of a device.

42. The method of any one of claims 28 to 38 or 39, wherein:

- the fluorophore of the first closed and open stem-loop oligonucleotides emits in the same colour region of the visible spectrum as the fluorophore of the second closed and open stem-loop oligonucleotides; and
- the fluorophore of the third closed and open stem-loop oligonucleotides emits in a different colour region of the visible spectrum than the fluorophore of the first and second closed and open stem-loop oligonucleotides.

43. The method of any one of claims 28 to 42, wherein the third temperature differs from the first temperature and/or 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, 60°C, 65°C, 70°C, 75°C, 80°C.

44. The method of any one of claims 28 to 43, wherein detection of a third fluorescent signal at the third temperature is indicative of the presence of the third target in the sample, and failure to detect a third fluorescent signal at the third temperature is indicative of the absence of the third target in the sample.

45. The method of any one of claims 1 to 44, wherein:

(i) determining a presence of the first fluorescent signal at the first temperature is indicative of the presence of the first target in the sample, and determining an absence of the first fluorescent signal is indicative of the absence of the first target in the sample; and

(ii) determining a presence of the second fluorescent signal at the second temperature is indicative of the presence of the second target in the sample, and determining an absence of the second fluorescent signal is indicative of the absence of the second target in the sample.

46. The method of any one of claims 1 to 44, wherein said determining the presence or absence of the first and second targets comprises a melt curve analysis using said first and second fluorescent signals.

47. The method of any one of claims 1 to 44, wherein part (c) comprises detecting levels of said first and second fluorescent signals at:

- a temperature equal to or above the second temperature; and
- a temperature equal to or above the first temperature and below the second temperature.

48. The method of claim 47, wherein part (c) further comprises detecting levels of said first and second fluorescent signals at a temperature below the second temperature.

49. The method of claim 47 or claim 48 wherein part (c) comprises detecting levels of said first and second fluorescent signals during and/or upon completion of a nucleic acid amplification reaction.

50. The method of any one of claims 47 to 49 further comprising generating a first target positive control fluorescent signal using a known concentration of the first target and/or a known concentration the first closed stem-loop oligonucleotide.

51. The method of any one of claims 47 to 50, further comprising generating a first target positive control fluorescent signal by repeating said method on a separate control sample comprising said first target.

52. The method of claim 51, wherein the control sample comprising the first target comprises a known concentration of the first target.

53. The method of claim 51 or claim 52, wherein the control sample comprising the first target further comprises said second target.

54. The method of any one of claims 46 to 53, further comprising generating a second target positive control fluorescent signal by repeating said method on a separate control sample comprising said second target.

55. The method of claim 54, wherein the control sample comprising the second target comprises a known concentration of the second target.

56. The method of claim 54 or claim 55, wherein said control sample further comprises said first target.

57. The method of any one of claims 46 to 53, further comprising generating a combined positive control fluorescent signal by repeating said method on a separate control sample comprising said first and said second targets.

58. The method of claim 57, wherein the combined control sample comprises a known concentration of the first target and/or a known concentration of the second target.

59. The method of any one of claims 50 to 58, further comprising normalising said first fluorescent signal and/or said second fluorescent signal using any said positive control fluorescent signal.

60. The method of any one of claims 46 to 59, further comprising generating a negative control fluorescent signal by repeating the method of any one of claims 1 to 3 on a separate negative control sample that does not contain:

(i) said first target; or

(ii) said second target; or

(iii) said first target or said second target.

61. The method of claim 60, further comprising normalising said first fluorescent signal and/or said second fluorescent signal using said negative control fluorescent signal.

62. The method of any one of claims 46 to 61, further comprising comparing said first and/or second fluorescent signals to a threshold value wherein:

- the threshold value is generated using fluorescent signals derived from a series of samples tested according to the method of any one of claims 1 to 3, and comprising any one or more of:

(i) a no template control and the first target

(ii) a no template control and the second target

(iii) a no template control, the first target, and the second target

to thereby determine said presence or absence of the first and second targets in the sample.

63. The method of claim 62, wherein the series of samples is tested using a known concentration of said first closed stem-loop oligonucleotide and/or a known concentration of said second closed stem-loop oligonucleotide.

64. The method of any one of claims 1 to 63, 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, 60°C, 65°C, 70°C, 75°C, 80°C.

65. The method of any one of claims 1 to 64, wherein any said amplicon thereof is produced by any one of more of polymerase chain reaction (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), and/or reverse transcription polymerase chain reaction (RT-PCR).

66. The method of any one of claims 1 to 65, wherein the sample is a biological sample obtained from a subject.

67. The method of any one of claims 1 to 65, wherein the method is performed *in vitro*.

68. The method of any one of claims 1 to 65, wherein the method is performed *ex vivo*.

69. A composition comprising:

first and second closed stem-loop oligonucleotides, wherein each of the closed stem-loop oligonucleotides comprise a double-stranded stem portion of hybridised nucleotides connected to a single-stranded loop portion of unhybridised nucleotides, wherein:

the number of hybridised nucleotides and/or the sequence of hybridised nucleotides in the stem portion of the first and second closed stem-loop oligonucleotide differs,

each of the double-stranded stem portions comprise a fluorophore molecule connected to one strand and a quencher molecule connected to an opposing strand, and

the single-stranded loop portions comprise a region that is capable of serving as a substrate for a catalytic nucleic acid, an exonuclease or an endonuclease,

wherein the fluorophore molecules emit in the same colour region of the visible spectrum, and the melting temperature (Tm) of the double-stranded stem portion of the first closed stem-loop oligonucleotide differs from the Tm of the double-stranded stem portion of the second closed stem-loop oligonucleotide.

70. The composition of claim 69, wherein the single-stranded loop portion of the first closed stem-loop oligonucleotide differs from the sequence of the single-stranded loop portion of the second closed stem-loop oligonucleotide.

71. The composition of claim 69 or claim 70, further comprising:

a first MNAzyme comprising substrate arms capable of hybridising to the closed single-stranded loop portion of the first closed stem-loop oligonucleotide; and

a second MNAzyme comprising substrate arms capable of hybridising to the single-stranded loop portion of the second closed stem-loop oligonucleotide.

72. The composition of claim 71, wherein:

the substrate arms of the first MNAzyme are hybridised to the single-stranded loop portion of the first closed stem-loop oligonucleotide; and

the substrate arms of the second MNAzyme are hybridised to the single-stranded loop portion of the second closed stem-loop oligonucleotide.

73. The composition of claim 72, wherein:

the first and/or second MNAzymes comprise an aptamer sequence bound to a target analyte, protein, compound or molecule and sensor arms hybridised to an oligonucleotide sequence; and

the first MNAzyme is designed to detect a different target than the second MNAzyme.

74. The composition of claim 72, wherein:

the first and/or second MNAzymes comprise sensor arms hybridised to a target sequence, and the first MNAzyme is designed to detect a different target than the second MNAzyme.

75. The composition of any one of claims 69 to 74, further comprising:

a first oligonucleotide sequence hybridised to said single-stranded loop portion of the first closed stem-loop oligonucleotide that differs from the sequence of the single-stranded loop portion of the second closed stem-loop oligonucleotide, thereby forming a double stranded sequence comprising said first oligonucleotide;

a second oligonucleotide sequence hybridised to said single-stranded loop portion of the second closed stem-loop oligonucleotide that differs from the sequence of the single-stranded loop portion of the first closed stem-loop oligonucleotide, thereby forming a double stranded sequence comprising said second oligonucleotide;

a restriction endonuclease associated with and capable of cleaving the double stranded sequence comprising said first oligonucleotide; and/or

a restriction endonuclease associated with and capable of cleaving the double stranded sequence comprising said second oligonucleotide.

76. The composition of any one of claims 69 to 75, further comprising:

a first double-stranded sequence comprising a first target oligonucleotide sequence hybridised to said single-stranded loop portion of the first closed stem-loop oligonucleotide that differs from the sequence of the single-stranded loop portion of the

second closed stem-loop oligonucleotide, thereby forming a first double stranded sequence comprising said first target oligonucleotide;

a second double-stranded sequence comprising a second target oligonucleotide sequence hybridised to said single-stranded loop portion of the second closed stem-loop oligonucleotide that differs from the sequence of the single-stranded loop portion of the first closed stem-loop oligonucleotide, thereby forming a double stranded sequence comprising said second target oligonucleotide;

a first primer oligonucleotide hybridised to the first target oligonucleotide upstream (5') relative to said first double-stranded sequence and a first enzyme comprising exonuclease activity (e.g. a polymerase, an exonuclease) associated with a loop portion of the first closed stem-loop oligonucleotide at or adjacent to a terminus of the first primer oligonucleotide; and/or

a second primer oligonucleotide hybridised to the second target oligonucleotide upstream (5') relative to said second double-stranded sequence and a second enzyme comprising exonuclease activity (e.g. a polymerase, an exonuclease) associated with a loop portion of the second closed stem-loop oligonucleotide at or adjacent to a terminus of the second primer oligonucleotide.

77. The composition of any one of claims 69 to 76, further comprising:

a first double-stranded sequence comprising a first target oligonucleotide sequence hybridised to said single-stranded loop portion of the closed first stem-loop oligonucleotide that differs from the sequence of the single-stranded loop portion of the second closed stem-loop oligonucleotide, thereby forming a first double-stranded sequence comprising said first target oligonucleotide;

a second double-stranded sequence comprising a second target oligonucleotide sequence hybridised to said single-stranded loop portion of the second closed stem-loop oligonucleotide that differs from the sequence of the single-stranded loop portion of the first closed stem-loop oligonucleotide, thereby forming a second double stranded sequence comprising said second target oligonucleotide;

wherein a first enzyme comprising exonuclease activity is associated with the first double-stranded sequence and a second enzyme comprising exonuclease activity is associated with the second double-stranded sequence.

78. The composition of any one of claims 69 to 77, further comprising:

a first DNAzyme and/or a first ribozyme hybridised to a loop portion of the first closed stem-loop oligonucleotide that differs from the sequence of the single-stranded loop portion of the second closed stem-loop oligonucleotide,

a second DNAzyme and/or a second ribozyme hybridised to a loop portion of the second closed stem-loop oligonucleotide that differs from the sequence of the single-stranded loop portion of the first closed stem-loop oligonucleotide,

cofactors for each said DNAzyme capable of rendering each said DNAzyme catalytically active, and thus induce cleavage activity of any said DNAzyme hybridised to the loop portion.

cofactors for each said ribozyme capable of rendering each said ribozyme catalytically active, and thus induce cleavage activity of any said ribozyme hybridised to the loop portion.

79. The composition of any one of claims 69 to 78, further comprising:

third closed stem-loop oligonucleotides, wherein the third closed stem-loop oligonucleotides comprise a double-stranded stem portion of hybridised nucleotides connected to a single-stranded loop portion of unhybridised nucleotides, wherein:

the number of hybridised nucleotides and/or the sequence of hybridised nucleotides in the stem portion of the third closed stem-loop oligonucleotides differs from those of the first and second closed stem-loop oligonucleotides, and

the double-stranded stem portion of the third closed stem-loop oligonucleotides comprises a fluorophore molecule connected to one strand and a quencher molecule connected to an opposing strand, wherein the fluorophore molecule of the third closed stem-loop oligonucleotide emits in a different colour region of the visible spectrum compared to the fluorophore molecules of the first and second closed stem-loop oligonucleotides.

80. The composition of any one of claims 69 to 78, further comprising:

third closed stem-loop oligonucleotides, wherein the third closed stem-loop oligonucleotides comprise a double-stranded stem portion of hybridised nucleotides connected to a single-stranded loop portion of unhybridised nucleotides, wherein:

the number of hybridised nucleotides and/or the sequence of hybridised nucleotides in the stem portion of the third closed stem-loop

oligonucleotides differs from those of the first and second closed stem-loop oligonucleotides, and

the double-stranded stem portion of the third closed stem-loop oligonucleotides comprises a fluorophore molecule connected to one strand and a quencher molecule connected to an opposing strand, wherein the fluorophore molecule of the third closed stem-loop oligonucleotide emits in a same colour region of the visible spectrum compared to the fluorophore molecules of the first and second closed stem-loop oligonucleotides.

81. The composition of any one of claims 69 to 78, further comprising:

third closed stem-loop oligonucleotides, wherein the third closed stem-loop oligonucleotides comprise a double-stranded stem portion of hybridised nucleotides connected to a single-stranded loop portion of unhybridised nucleotides, wherein:

the number of hybridised nucleotides and/or the sequence of hybridised nucleotides in the stem portion of the third closed stem-loop oligonucleotides is the same those of the first or second closed stem-loop oligonucleotides, and

the double-stranded stem portion of the third closed stem-loop oligonucleotides comprises a fluorophore molecule connected to one strand and a quencher molecule connected to an opposing strand, wherein the fluorophore molecule of the third closed stem-loop oligonucleotide emits in a different colour region of the visible spectrum compared to the fluorophore molecules of the first and second closed stem-loop oligonucleotides.