

CLAIMS

1. A method for determining the presence or absence of first and second targets 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 second targets with:

- a first oligonucleotide for detection of the first target, and comprising a first detection moiety capable of generating a first detectable signal;

- an intact stem-loop oligonucleotide for detection of the second target, and 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, wherein the stem portion comprises a second detection moiety capable of generating a second detectable signal,

wherein the first and second detection moieties are capable of generating detectable signals that cannot be differentiated at a single temperature using a single type of detector; and

- a first enzyme capable of digesting one or more of the unhybridised nucleotides of the intact stem-loop oligonucleotide only when the second target is present in the sample;

(b) treating the mixture under conditions suitable for:

- the first target to induce a modification to the first oligonucleotide thereby enabling the first detection moiety to generate a first detectable signal,

- digestion of one or more of the unhybridised nucleotides of the intact stem-loop oligonucleotide by the first enzyme, only when the second target is present in the sample, to thereby break the single-stranded loop portion and provide a split stem-loop oligonucleotide;

- (c) measuring:
 - a background signal provided by the first and the second detection moieties in the mixture, or, in a control mixture;
 - (d) determining whether at one or more timepoints during or after said treating:
 - a first detectable signal arising from said modification is generated at a first temperature which differs from the background signal and is indicative of the presence of the first target in the sample;
 - a second detectable signal is generated at a second temperature which differs from the background signal and is indicative of the presence of the second target in the sample;
 - wherein:
 - at the first temperature the second detectable signal does not differ from the background signal, and
 - at the second temperature:
 - if present, strands of the double-stranded stem portion of the split stem-loop oligonucleotide are partially or completely dissociated enabling the second detection moiety to provide the second detectable signal; and
 - if present, strands of the double-stranded stem portion of the intact stem-loop oligonucleotide cannot dissociate thereby preventing the second detectable moiety from providing the second detectable signal.
2. The method of claim 1, wherein said determining in part (d) comprises:
- using a predetermined threshold value to determine if the first detectable signal arising from said modification 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.
3. The method of claim 1 or claim 2, wherein the control mixture does not comprise:
- the first target; or
 - the second target; or
 - the first and second targets,

but is otherwise equivalent to the mixture.

4. The method of any one of claims 1 to 3, wherein the control mixture comprises a predetermined amount of:

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

but is otherwise equivalent to the mixture.

5. The method of any one of claims 1 to 4, wherein:

- the modification to the first oligonucleotide enables the first detection moiety to provide the first detectable signal at or below the first temperature; and
- generation of the first detectable signal is reversible.

6. The method of claim 5, wherein:

- part (c) comprises measuring:
 - a first background signal at or within 1°C, 2°C, 3°C, 4°C, or 5°C of a first temperature, and a second background signal at or within 1°C, 2°C, 3°C, 4°C, or 5°C of a second temperature;

provided by the first and the second detection moieties in the mixture, or, in the control mixture; and

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

a first detectable signal arising from said modification 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;

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.

7. The method of claim 5 or claim 6, wherein:

- the first target is a nucleic acid sequence;
- the first oligonucleotide is a stem-loop oligonucleotide comprising a double-stranded stem portion of hybridised nucleotides on opposing strands of which are

linked by an unbroken single-stranded loop portion of unhybridised nucleotides of which all or a portion is/are complementary to the first target; and

- the modification of the first oligonucleotide is a conformational change arising from hybridisation of the target to the single-stranded loop portion of the first oligonucleotide by complementary base pairing.

8. The method of claim 7, wherein:

- the conformational change is dissociation of strands in the double-stranded stem portion of the first oligonucleotide arising from said hybridisation of the target to the single-stranded loop portion of the first oligonucleotide by complementary base pairing.

9. The method of claim 7 or claim 8, wherein:

- the stem portion of the first oligonucleotide has a melting temperature (T_m) that is: below the T_m of a double-stranded duplex formed from said hybridisation of the target to the single-stranded loop portion of the first oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligonucleotide;

- said double-stranded duplex has a T_m that is: above the T_m of the stem portion of the intact stem-loop oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligonucleotide;

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

- the first temperature is below the T_m of: said double-stranded duplex, the stem portion of the first oligonucleotide, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;

- the second temperature is below the T_m of: said double-stranded duplex, the stem portion of the first oligonucleotide, and the stem portion of the intact stem-loop oligonucleotide; and is above the T_m of the stem portion of the split stem-loop oligonucleotide; and

- the first temperature is below the second temperature.

10. The method of claim 7 or claim 8, wherein:

- the stem portion of the first oligonucleotide has a melting temperature (T_m) that is: below the T_m of a double-stranded duplex formed from said hybridisation of

the target to the single-stranded loop portion of the first oligonucleotide, below the T_m of the stem portion of the intact stem-loop oligonucleotide;

- said double-stranded duplex has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: said double-stranded duplex, the stem portion of the first oligonucleotide, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is above the T_m of: the stem portion of the first oligonucleotide, and the stem portion of the split stem-loop oligonucleotide; and is below the T_m of: said double-stranded duplex, and the stem portion of the intact stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

11. The method of claim 7 or claim 8, wherein:

- the stem portion of the first oligonucleotide has a melting temperature (T_m) that is: below the T_m of a double-stranded duplex formed from said hybridisation of the target to the single-stranded loop portion of the first oligonucleotide, below the T_m of the stem portion of the intact stem-loop oligonucleotide, and below the T_m of the stem portion of the split stem-loop oligonucleotide;
- said double-stranded duplex has a T_m that is: below the T_m of the stem portion of the intact stem-loop oligonucleotide;
- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: said double-stranded duplex, the stem portion of the first oligonucleotide, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is above the T_m of: said double-stranded duplex, the stem portion of the first oligonucleotide, and the stem portion of the split stem-loop oligonucleotide; and is below the T_m of: the stem portion of the intact stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

12. The method of claim 7 or claim 8, wherein:

- the stem portion of the first oligonucleotide has a melting temperature (T_m) that is: below the T_m of a double-stranded duplex formed from said hybridisation of the target to the single-stranded loop portion of the first oligonucleotide, above the T_m of the stem portion of the intact stem-loop oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligonucleotide;
- said double-stranded duplex has a T_m that is: above the T_m of the stem portion of the intact stem-loop oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the stem portion of the first oligonucleotide and said double-stranded duplex; and above the T_m of: the stem portion of the intact stem-loop oligonucleotide and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is below the T_m of: the stem portion of the first oligonucleotide, said double-stranded duplex, and the stem portion of the intact stem-loop oligonucleotide; and is above the T_m of the stem portion of the split stem-loop oligonucleotide; and
- the first temperature is above the second temperature.

13. The method of any one of claims 7 to 12, wherein:

- the T_m of the stem portion of the first oligonucleotide is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of said double-stranded duplex; and/or
- the T_m of the stem portion of the intact stem-loop oligonucleotide is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide; and/or
- the first temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of: the stem portion of the first oligonucleotide, and/or said double-stranded duplex; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the stem portion of the intact stem-loop oligonucleotide; and/or

- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide.

14. The method of claim 5 or claim 6, wherein:

- the first target is a nucleic acid sequence;
- the first oligonucleotide is a stem-loop oligonucleotide comprising:
 - a double-stranded stem portion of hybridised nucleotides, opposing strands of which are linked by a single-stranded loop portion of unhybridised nucleotides, all or a portion of which is/are complementary to the first target, and a second single-stranded portion extending from one of said opposing strands in a 3' direction and terminating with a sequence that is complementary to a portion of the first target, and
 - a blocker molecule preceding said sequence that is complementary to the portion of the first target;
- the mixture further comprises a polymerase;
- said treating the mixture comprises:
 - hybridising the second single-stranded portion to the first target by complementary base pairing;
 - extending the second single-stranded portion using the polymerase and the first target as a template sequence to provide a double-stranded nucleic acid, wherein said blocker molecule prevents the polymerase extending the first target using the stem portion of the first oligonucleotide as a template; and
 - denaturing the double-stranded nucleic acid and hybridising the second single-stranded portion extended by the polymerase to the single-stranded loop portion of the first oligonucleotide by complementary base pairing to produce a signaling duplex and thereby provide said modification to the first oligonucleotide enabling the first detection moiety to provide the first detectable signal.

15. The method of claim 14, wherein:

- the stem portion of the first oligonucleotide has a melting temperature (T_m) that is: below the T_m of the signaling duplex and above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the signaling duplex has a T_m that is: above the T_m of the stem portion of the intact stem-loop oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligo nucleotide;

- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the signaling duplex, the stem portion of the first oligonucleotide, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is below the T_m of: the signaling duplex, the stem portion of the first oligonucleotide, and the stem portion of the intact stem-loop oligonucleotide; and is above the T_m of the stem portion of the split stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

16. The method of claim 14, wherein:

- the stem portion of the first oligonucleotide has a melting temperature (T_m) that is: below the T_m of the signaling duplex, below the T_m of the stem portion of the intact stem-loop oligonucleotide;
- the signaling duplex has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the signaling duplex, the stem portion of the first oligonucleotide, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is above the T_m of: the stem portion of the first oligonucleotide, and the stem portion of the split stem-loop oligonucleotide; and is below the T_m of: the signaling duplex, and the stem portion of the intact stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

17. The method of claim 14, wherein:

- the stem portion of the first oligonucleotide has a melting temperature (T_m) that is: below the T_m of the signaling duplex, below the T_m of the stem portion of the intact stem-loop oligonucleotide, and below the T_m of the stem portion of the split stem-loop oligonucleotide;
- the signaling duplex has a T_m that is: below the T_m of the stem portion of the intact stem-loop oligonucleotide,

- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the signaling duplex, the stem portion of the first oligonucleotide, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is above the T_m of: the signaling duplex, the stem portion of the first oligonucleotide, and the stem portion of the split stem-loop oligonucleotide; and is below the T_m of: the stem portion of the intact stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

18. The method of claim 14, wherein:

- the stem portion of the first oligonucleotide has a melting temperature (T_m) that is: below the T_m of the signaling duplex, above the T_m of the stem portion of the intact stem-loop oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the signaling duplex has a T_m that is: above the T_m of the stem portion of the intact stem-loop oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the stem portion of the first oligonucleotide and the signaling duplex; and above the T_m of: the stem portion of the intact stem-loop oligonucleotide and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is below the T_m of: the stem portion of the first oligonucleotide, the signaling duplex, and the stem portion of the intact stem-loop oligonucleotide; and is above the T_m of the stem portion of the split stem-loop oligonucleotide; and
- the first temperature is above the second temperature.

19. The method of any one of claims 14 to 18, wherein:

- the T_m of the stem portion of the first oligonucleotide is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the signaling duplex; and/or

- the T_m of the stem portion of the intact stem-loop oligonucleotide is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide; and/or
- the first temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of: the stem portion of the first oligonucleotide, and/or the signaling duplex; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the stem portion of the intact stem-loop oligonucleotide; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide.

20. The method of any one of claims 5 to 19, wherein:

- the first detection moiety is a fluorophore and the modification increases its distance from a quencher molecule.

21. The method of claim 20, wherein:

- the first oligonucleotide comprises the quencher molecule.

22. The method of claim 21, wherein:

- the fluorophore and the quencher molecule are located on opposing strands of the double-stranded stem portion of the first oligonucleotide.

23. The method of claim 5 or claim 6, wherein:

- the first target is a nucleic acid sequence;
- the first oligonucleotide comprises:

a first double-stranded portion of hybridised nucleotides, a first strand of which extends into a single-stranded portion terminating with a complementary sequence capable of hybridising to a portion of the first target, wherein the first strand comprises a blocker molecule preceding said complementary sequence;

- the mixture further comprises a polymerase;
- said treating the mixture comprises:
hybridising said complementary sequence of the single-stranded portion to a portion of the first target by complementary base pairing;

extending the complementary sequence using the polymerase and the first target as a template sequence to provide a second double-stranded portion, wherein said blocker molecule prevents the polymerase extending the first target using the first strand of the said first double-stranded portion as a template;

denaturing the first and second double-stranded portions; and

hybridising the complementary sequence extended by the polymerase to the first strand of the first double-stranded portion by complementary base pairing to produce a signaling duplex and thereby provide said modification to the first oligonucleotide enabling the first detection moiety to provide the first detectable signal.

24. The method of claim 23, wherein:

- the first double-stranded portion has a melting temperature (T_m) that is: below the T_m of the signaling duplex, and above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the signaling duplex has a T_m that is: above the T_m of the stem portion of the intact stem-loop oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the signaling duplex, the first double-stranded portion, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is below the T_m of: the signaling duplex, the first double-stranded portion, and the stem portion of the intact stem-loop oligonucleotide; and is above the T_m of the stem portion of the split stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

25. The method of claim 23, wherein:

- the first double-stranded portion has a melting temperature (T_m) that is: below the T_m of the signaling duplex, below the T_m of the stem portion of the intact stem-loop oligonucleotide;
- the signaling duplex has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;

- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the signaling duplex, the first double-stranded portion, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is above the T_m of: the first double-stranded portion, and the stem portion of the split stem-loop oligonucleotide; and is below the T_m of: the signaling duplex, and the stem portion of the intact stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

26. The method of claim 23, wherein:

- the first double-stranded portion has a melting temperature (T_m) that is: below the T_m of the signaling duplex, below the T_m of the stem portion of the intact stem-loop oligonucleotide, and below the T_m of the stem portion of the split stem-loop oligonucleotide;
- the signaling duplex has a T_m that is below the T_m of the stem portion of the intact stem-loop oligonucleotide;
- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the signaling duplex, the first double-stranded portion, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is above the T_m of: the signaling duplex, the first double-stranded portion, and the stem portion of the split stem-loop oligonucleotide; and is below the T_m of: the stem portion of the intact stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

27. The method of claim 23, wherein:

- the first double-stranded portion has a melting temperature (T_m) that is: below the T_m of the signaling duplex, above the T_m of the stem portion of the intact stem-loop oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the signaling duplex has a T_m that is: above the T_m of the stem portion of the intact stem-loop oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligonucleotide;

- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the first double-stranded portion and the signaling duplex; and above the T_m of: the stem portion of the intact stem-loop oligonucleotide and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is below the T_m of: the first double-stranded portion, the signaling duplex, and the stem portion of the intact stem-loop oligonucleotide; and is above the T_m of the stem portion of the split stem-loop oligonucleotide; and
- the first temperature is above the second temperature.

28. The method of any one of claims 23 to 27, wherein:

- the T_m of the first double-stranded portion is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the signaling duplex; and/or
- the T_m of the stem portion of the intact stem-loop oligonucleotide is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide; and/or
- the first temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of: the first double-stranded portion, and/or the signaling duplex; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the stem portion of the intact stem-loop oligonucleotide; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide.

29. The method of any one of claims 23 to 28, wherein:

- the first detection moiety is a fluorophore and the modification increases its distance from a quencher molecule.

30. The method of claim 29, wherein:

- the first oligonucleotide comprises the quencher molecule.

31. The method of claim 30, wherein:
- the fluorophore and the quencher molecule are located on opposing strands of the first double-stranded portion.
32. The method of claim 5 or claim 6, wherein:
- the first target is a nucleic acid sequence;
 - the mixture further comprises:
 - a first primer complementary to a first sequence in the first target,
 - a second oligonucleotide comprising a component complementary to a second sequence in the first target that differs from the first sequence, and a tag portion that is not complementary to the first target,
 - a first polymerase comprising exonuclease activity, and
 - optionally a second polymerase, and
 - said treating the mixture comprises:
 - suitable conditions to hybridise the first primer and the second oligonucleotide to the first target,
 - extending the first primer using the first polymerase and the target as a template to thereby cleave off the tag portion,
 - hybridising the cleaved tag portion to the first oligonucleotide by complementary base pairing,
 - and extending the tag portion using the first or second polymerase and the first oligonucleotide as a template to generate a double-stranded sequence comprising the first oligonucleotide thereby providing said modification to the first oligonucleotide and enabling the first detection moiety to provide the first detectable signal.
33. The method of claim 32, wherein:
- the double-stranded sequence has a T_m that is: above the T_m of the stem portion of the intact stem-loop oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligonucleotide;
 - the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
 - the first temperature is below the T_m of: the double-stranded sequence, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;

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

34. The method of claim 32, wherein:

- the double-stranded sequence has a T_m that is: above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the double-stranded sequence, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is above the T_m of: and the stem portion of the split stem-loop oligonucleotide; and is below the T_m of: the double-stranded sequence, and the stem portion of the intact stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

35. The method of claim 32, wherein:

- the double-stranded sequence has a T_m that is: below the T_m of the stem portion of the intact stem-loop oligonucleotide;
- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the double-stranded sequence, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is above the T_m of: the double-stranded sequence, and the stem portion of the split stem-loop oligonucleotide; and is below the T_m of: the stem portion of the intact stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

36. The method of claim 32, wherein:

- the double-stranded sequence has a T_m that is: above the T_m of the stem portion of the intact stem-loop oligonucleotide, and above the T_m of the stem portion of the split stem-loop oligonucleotide;

- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the double-stranded sequence; and above the T_m of: the stem portion of the intact stem-loop oligonucleotide and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is below the T_m of: the double-stranded sequence and the stem portion of the intact stem-loop oligonucleotide; and is above the T_m of the stem portion of the split stem-loop oligonucleotide; and
- the first temperature is above the second temperature.

37. The method of any one of claims 32 to 36, wherein:

- the T_m of the stem portion of the intact stem-loop oligonucleotide is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide; and/or
- the first temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the double-stranded sequence; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the stem portion of the intact stem-loop oligonucleotide; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide.

38. The method of any one of claims 32 to 37, wherein:

- the first oligonucleotide comprises a fluorophore and a quencher molecule, and
- said extending the tag portion increases the distance between the fluorophore and the quencher molecule.

39. The method of claim 5 or claim 6, wherein:

- the first target is a nucleic acid sequence;
- the first oligonucleotide is complementary to a first portion of the target;
- the mixture further comprises a further oligonucleotide complementary to a second portion the first target, wherein the first and second portions of the first target flank one another but do not overlap;

- said treating the mixture comprises:
 - forming a duplex structure comprising:
 - (iii) a first double-stranded component by hybridising the first oligonucleotide to the target by complementary base pairing, and
 - (iv) a second double-stranded component by hybridising the further oligonucleotide to the target by complementary base pairing,
 - thereby bringing the first and further oligonucleotides into proximity, and providing said modification to the first oligonucleotide enabling the first detection moiety to provide the first detectable signal.

40. The method of claim 39, wherein:

- the duplex structure has a T_m that is below the T_m of the stem portion of the intact stem-loop oligonucleotide;
- the stem portion of the intact stem-loop oligonucleotide has a T_m that is above the T_m of the stem portion of the split stem-loop oligonucleotide;
- the first temperature is below the T_m of: the duplex structure, the stem portion of the intact stem-loop oligonucleotide, and the stem portion of the split stem-loop oligonucleotide;
- the second temperature is above the T_m of: the duplex structure, and the stem portion of the split stem-loop oligonucleotide; and is below the T_m of: the stem portion of the intact stem-loop oligonucleotide; and
- the first temperature is below the second temperature.

41. The method of claim 39 of claim 40, wherein:

- the T_m of the stem portion of the intact stem-loop oligonucleotide is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide; and/or
- the first temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the duplex structure; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the stem portion of the intact stem-loop oligonucleotide; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide.

42. The method of any one of claims 39 to 41, wherein:

- the first detectable moiety is a fluorophore and the further oligonucleotide comprises a quencher;
- said forming of the duplex structure further brings the fluorophore and quencher into proximity; and
- said detectable signal is a decrease in fluorescence provided by the first detection moiety.

43. The method of claim 5 or claim 6, wherein:

- the first target is a nucleic acid sequence;
- the first detection moiety is: a nanoparticle, a metallic nanoparticle, a noble metal nanoparticle, an alkali metal nanoparticle, a gold nanoparticle, or a silver nanoparticle; to which the first oligonucleotide is bound;
- said treating the mixture comprises:
 - hybridising the first target to the first oligonucleotide to thereby induce the modification to the first oligonucleotide enabling the first detection moiety to provide a first detectable signal indicative of the presence of the first target in the sample;
 - wherein the first detectable signal is:
 - (i) a change in refractive index,
 - (ii) a change in colour; and/or
 - (iii) a change in absorption spectrum,
 - arising from the first detection moiety following said modification of the first oligonucleotide.

44. The method of claim 5 or claim 6, wherein:

- the first target is a nucleic acid sequence;
- the first detection moiety is an electrochemical agent to which the first oligonucleotide is bound;
- said treating the mixture comprises:
 - hybridising the first target to the first oligonucleotide to thereby induce or facilitate the modification to the first oligonucleotide enabling the first detection moiety to provide a first detectable signal indicative of the presence of the first target in the sample;

wherein the first detectable signal is a change in electrochemical signal arising from the first detection moiety following said modification of the first oligonucleotide.

45. The method of claim 44, wherein:

- the electrochemical agent is selected from any one or more of a nanoparticle, Methylene blue, Toluene blue, Oracet Blue, Hoechst 33258, [Ru(phen)₃]²⁺, ferrocene, and/or daunomycin.

46. The method of any one of claims 5 to 19, 23 to 28, 32 to 37, and 39 to 41 wherein:

- the first detection moiety is a nanoparticle, a metallic nanoparticle, a noble metal nanoparticle, an alkali metal nanoparticle, a gold nanoparticle, or a silver nanoparticle; to which the first oligonucleotide is bound; and

- the first detectable signal is:

- (i) a change in refractive index,
- (ii) a change in colour; and/or
- (iii) a change in absorption spectrum,

arising from the first detection moiety following said modification of the first oligonucleotide.

47. The method of any one of claims 5 to 19, 23 to 28, 32 to 37, and 39 to 41 wherein:

- the first detection moiety is an electrochemical agent to which the first oligonucleotide is bound; and

- the first detectable signal is a change in electrochemical signal arising from the first detection moiety following said modification of the first oligonucleotide.

48. The method of claim 47, wherein:

- the electrochemical agent is selected from any one or more of a nanoparticle, Methylene blue, Toluene blue, Oracet Blue, Hoechst 33258, [Ru(phen)₃]²⁺, ferrocene, and/or daunomycin.

49. The method of any one of claims 43 to 48, wherein:

- the second detection moiety is a nanoparticle, a metallic nanoparticle, a noble metal nanoparticle, an alkali metal nanoparticle, a gold nanoparticle, or a silver nanoparticle; to which the intact stem-loop oligonucleotide is bound; and
- the second detectable signal is:
 - (i) a change in refractive index,
 - (ii) a change in colour; and/or
 - (iii) a change in absorption spectrum,arising from said strands of the double-stranded stem portion of the split stem-loop oligonucleotide dissociating.

50. The method of any one of any one of claims 43 to 48, wherein:

- the second detection moiety is an electrochemical agent to which the intact stem-loop oligonucleotide is bound; and
- the second detectable signal is a change in electrochemical signal arising from said strands of the double-stranded stem portion of the split stem-loop oligonucleotide dissociating.

51. The method of claim 50, wherein:

- the electrochemical agent is selected from any one or more of a nanoparticle, Methylene blue, Toluene blue, Oracet Blue, Hoechst 33258, [Ru(phen)₃]²⁺, ferrocene, and/or daunomycin.

52. The method of any one of claims 20 to 22, 29 to 31, 38, and 42, wherein:

- the second detection moiety is a fluorophore, and
- the second detectable signal provided by said strands of the double-stranded stem portion of the split stem-loop oligonucleotide dissociating increases the distance of the fluorophore from a quencher molecule.

53. The method of claim 52, wherein:

- the fluorophore and quencher molecule are located on opposing strands of the double-stranded stem portion of the split stem-loop oligonucleotide.

54. The method of any one of claims 1 to 4, wherein:

- generation of the first detectable signal is not reversible;

- the modification to the first oligonucleotide enables the first detection moiety to provide the first detectable signal at or below the first temperature; and
- the first detectable signal provided at or below the first temperature remains detectable at the second temperature.

55. The method of claim 54, wherein:

- part (c) comprises measuring:
 - (i) a first background signal at or within 1°C, 2°C, 3°C, 4°C, or 5°C of a first temperature, and a second background signal at or within 1°C, 2°C, 3°C, 4°C, or 5°C of a second temperature, and/or
 - (ii) a third background signal at a third temperature;provided by the first and the second detection moieties in the mixture, or, in a control mixture; and
- part (d) comprises determining whether at one or more timepoints during or after said treating:
 - (i) a first detectable signal arising from said modification is generated at the first temperature which differs from the first or third background signal, wherein:
 - at the first temperature the second detectable signal does not differ from the first or third background signal, and
 - detection of a difference between the first detectable signal and the first or third background signal is indicative of said modification of the first oligonucleotide and the presence of the first target in the sample; and
 - (ii) a second detectable signal is generated at the second temperature which differs from the second or third background signal and is indicative of the presence of the second target in the sample.

56. The method of claim 55, wherein:

- when a first target is present in the sample, said determining whether a second detectable signal is generated at the second temperature comprises compensating for the first detectable signal present when measuring the second detectable signal.

57. The method of claim 55 or claim 56, wherein:

- the first signal that differs from the first background signal is generated,
- the second signal that differs from the second background signal is generated, and

- the second detectable signal differs from the second background signal to a greater extent than the first detectable signal differs from the first background signal, thereby indicating that the second target is present in the sample.

58. The method of claim 57, wherein:

- the first temperature is below: the second temperature, the T_m of the double-stranded stem portion of the intact stem-loop oligonucleotide, and the T_m of the stem portion of the split stem-loop oligonucleotide.

59. The method of claim 57, wherein:

- the first temperature is higher than: the second temperature, the T_m of the stem portion of the intact stem-loop oligonucleotide, and the T_m of the stem portion of the split stem-loop oligonucleotide.

60. The method of claim 55, wherein:

- the first signal that differs from the third background signal is generated,
- the second signal that differs from the third background signal is generated, and
- the second signal differs from the third background signal to a greater extent than the first signal differs from the third background signal, thereby indicating that the second target is present in the sample.

61. The method of claim 55, wherein:

- the second temperature is higher than the first temperature,
- the third temperature is lower the T_m of the double-stranded stem portion of the intact stem-loop oligonucleotide,
- the first detectable signal that differs from the third background signal is generated,
- the second detectable signal that differs from the third background signal is generated, and
- the second detectable signal differs from the third background signal to a greater extent than the first signal differs from the third background signal, thereby indicating that the second target is present in the sample.

62. The method of any one of claims 55 to 61 wherein:

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

63. The method of claim 62, wherein:

- the T_m of the stem portion of the intact stem-loop oligonucleotide is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide; and/or
- the first temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the second temperature; and/or
- the first temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the stem portion of the split stem-loop oligonucleotide; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the stem portion of the intact stem-loop oligonucleotide.

64. The method of claim 62 or claim 63, comprising:

- measuring said third background signal, wherein the third temperature is below the second temperature.

65. The method of claim 64, wherein:

- the third temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the second temperature.

66. The method of any one of claims 55 to 61 wherein:

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

67. The method of claim 66, wherein:

- the T_m of the stem portion of the intact stem-loop oligonucleotide is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide; and/or
- the first temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the second temperature; and/or
- the first temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide; and/or
- the first temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the intact stem-loop oligonucleotide; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, above the T_m of the stem portion of the split stem-loop oligonucleotide; and/or
- the second temperature is between 1°C and 10°C, 1°C and 5°C, 5°C and 10°C, or more than 10°C, below the T_m of the stem portion of the intact stem-loop oligonucleotide.

68. The method of any one of claims 54 to 67, wherein:

- the first oligonucleotide is a substrate for a multi-component nucleic acid enzyme (MNAzyme);
- the mixture further comprises:
 - an MNAzyme capable of cleaving the first oligonucleotide when the first target is present in the sample; and
- said treating the mixture further comprises:

binding of the MNzyme to the first target and hybridisation of the substrate arms of the MNzyme to the first oligonucleotide by complementary base pairing to facilitate cleavage of the first oligonucleotide thereby providing said modification to the first oligonucleotide and enabling the first detection moiety to provide the first detectable signal.

69. The method of claim 68, wherein:

- the first target is a nucleic acid sequence; and
- said treating the reaction mixture further comprises:

hybridising the first target to the sensor arms of the MNzyme by complementary base pairing to thereby facilitate assembly of the MNzyme.

70. The method of any one of claims 54 to 67, wherein:

- the first oligonucleotide is a substrate for an aptzyme;
- the first target is an analyte, protein, compound or molecule;
- the mixture further comprises an aptzyme comprising an aptamer capable of binding to the first target; and
- said treating the mixture further comprises:

binding of the aptzyme to the first target and the first oligonucleotide to facilitate cleavage of the first oligonucleotide thereby providing said modification to the first oligonucleotide and enabling the first detection moiety to generate the first detectable signal.

71. The method of any one of claims 54 to 67, wherein:

- the first target is a nucleic acid sequence;
- the first oligonucleotide comprises a sequence that is complementary to the first target,
- the mixture further comprises:
 - a primer complementary to a portion of the first target, and
 - a polymerase with exonuclease activity;
- said treating the mixture comprises:
 - hybridising the primer to the first target by complementary base pairing,
 - hybridising the first oligonucleotide to the first target by complementary base pairing

extending the primer using the polymerase and the first target as a template sequence to thereby digest the first oligonucleotide and provide said modification to the first oligonucleotide enabling the first detection moiety to generate the first detectable signal.

72. The method any one of claims 54 to 67, wherein:

- the first target is a nucleic acid sequence;
- the mixture further comprises:

a restriction endonuclease capable of digesting a double-stranded duplex comprising the first target; and

- said treating the mixture comprises:

hybridising the first oligonucleotide to the first target by complementary base pairing to thereby form a double-stranded duplex,

digesting the duplex using the restriction endonuclease to thereby provide said modification to the first oligonucleotide and enabling the first detection moiety to provide the first detectable signal.

73. The method of claim 72, wherein:

- the restriction endonuclease is a nicking endonuclease capable of associating with and cleaving a strand of said double-stranded duplex, and said strand comprises all or a portion of the first oligonucleotide.

74. The method of any one of claims 54 to 67, 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 cofactor to the DNAzyme or ribozyme to render it catalytically active,

hybridisation of the DNAzyme or ribozyme to the first oligonucleotide by complementary base pairing, and

catalytic activity of the DNAzyme or ribozyme to thereby digest the first oligonucleotide and thereby provide said modification to the first oligonucleotide enabling the first detection moiety to provide the first detectable signal.

wherein:

the first target is the co-factor.

75. The method of claim 74, wherein the co-factor is a metal ion, or a metal ion selected from: Mg^{2+} , Mn^{2+} , Ca^{2+} , Pb^{2+} .

76. The method of any one of claims 54 to 75, wherein:

- the first detection moiety is a fluorophore and the modification to the first oligonucleotide increases the distance of the fluorophore from a quencher molecule.

77. The method of claim 76, wherein:

- the first oligonucleotide comprises the quencher molecule.

78. The method of claim 76 or claim 77, wherein:

- the second detection moiety is a fluorophore, and
- the second detectable signal provided by said strands of the double-stranded stem portion of the split stem-loop oligonucleotide dissociating increases the distance of the fluorophore from a quencher molecule.

79. The method of claim 78, wherein:

- the fluorophore and quencher molecule are located on opposing strands of the double-stranded stem portion of the split stem-loop oligonucleotide.

80. The method of any one of claims 54 to 79, wherein:

- the first detection moiety is a nanoparticle, a metallic nanoparticle, a noble metal nanoparticle, an alkali metal nanoparticle, a gold nanoparticle, or a silver nanoparticle; to which the first oligonucleotide is bound; and

- the first detectable signal is:

- (i) a change in refractive index,
- (ii) a change in colour; and/or
- (iii) a change in absorption spectrum,

arising from the first detection moiety following said modification of the first oligonucleotide.

81. The method of any one of claims 54 to 79, wherein:

- the first detection moiety is an electrochemical agent to which the first oligonucleotide is bound; and
- the first detectable signal is a change in electrochemical signal arising from the first detection moiety following said modification of the first oligonucleotide.

82. The method of claim 81, wherein:

- the electrochemical agent is selected from any one or more of a nanoparticle, Methylene blue, Toluene blue, Oracet Blue, Hoechst 33258, [Ru(phen)₃]²⁺, ferrocene, and/or daunomycin.

83. The method of any one of claims 80 to 82, wherein:

- the second detection moiety is a nanoparticle, a metallic nanoparticle, a noble metal nanoparticle, an alkali metal nanoparticle, a gold nanoparticle, or a silver nanoparticle; to which the intact stem-loop oligonucleotide is bound; and
- the second detectable signal is:
 - (i) a change in refractive index,
 - (ii) a change in colour; and/or
 - (iii) a change in absorption spectrum,

arising from said strands of the double-stranded stem portion of the split stem-loop oligonucleotide dissociating.

84. The method of any one of any one of claims 80 to 82, wherein:

- the second detection moiety is an electrochemical agent to which the intact stem-loop oligonucleotide is bound; and
- the second detectable signal is a change in electrochemical signal arising from said strands of the double-stranded stem portion of the split stem-loop oligonucleotide dissociating.

85. The method of claim 84, wherein:

- the electrochemical agent is selected from any one or more of a nanoparticle, Methylene blue, Toluene blue, Oracet Blue, Hoechst 33258, [Ru(phen)₃]²⁺, ferrocene, and/or daunomycin.

86. The method of any one of claims 1 to 85, wherein the intact stem-loop oligonucleotide is not hybridised to the second target during said digestion of the one or

more unhybridised nucleotides of the intact stem-loop oligonucleotide by the first enzyme.

87. The method of any one of claims 1 to 86, wherein:

- the first enzyme is a first MNAzyme, and
- said treating the mixture comprises:

binding of the first MNAzyme to the second target and hybridisation of substrate arms of said first MNAzyme to the loop portion of the intact stem-loop oligonucleotide, to thereby digest the one or more unhybridised nucleotides of the intact stem-loop oligonucleotide and provide the split stem-loop oligonucleotide.

88. The method of claim 87, wherein:

- the second target is a nucleic acid sequence; and
- said treating the mixture further comprises:

hybridising the second target to the sensor arms of the first MNAzyme by complementary base pairing to thereby facilitate assembly of the first MNAzyme.

89. The method of any one of claims 1 to 86, wherein:

- the second target is an analyte, protein, compound or molecule;
- the first enzyme is an aptazyme comprising an aptamer capable of binding to the second target; and
- binding of the second target to the aptamer is capable of rendering the first enzyme catalytically active.

90. The method of claim 89, wherein:

- the first enzyme is any one of an: apta-DNAzyme, apta-ribozyme, apta-MNAzyme.

91. The method of any one of claims 1 to 86, wherein:

- the second target is an analyte, protein, compound or molecule;
- the first oligonucleotide is a substrate for an aptazyme;
- the first enzyme is an aptazyme comprising an aptamer portion capable of binding to the second target, and a nucleic acid enzyme portion capable of digesting the one or more unhybridised nucleotides of the intact stem-loop oligonucleotide

- said treating the mixture further comprises:

binding the second target to the aptamer portion of the aptazyme to facilitate activation of catalytic activity of the nucleic acid enzyme portion, and hybridising the intact stem-loop oligonucleotide to the active nucleic acid enzyme portion to thereby digest the one or more unhybridised nucleotides of the intact stem-loop oligonucleotide.

92. The method of any one of claims 1 to 85, wherein:

- the second target is a nucleic acid sequence; and
- the first enzyme is a first restriction endonuclease, and said treating the mixture comprises:

using conditions suitable for hybridisation of the second target to the single-stranded loop portion of the intact stem-loop oligonucleotide by complementary base pairing to form a double-stranded sequence for the first restriction endonuclease to associate with and digest the one or more unhybridised nucleotides of the single-stranded loop portion thereby forming the split stem-loop oligonucleotide.

93. The method of claim 92, wherein:

- the first restriction endonuclease is a first nicking endonuclease capable of associating with and cleaving a strand of said double-stranded sequence for the first restriction endonuclease, and said strand comprises all or a portion of the single-stranded loop portion of the intact stem-loop oligonucleotide.

94. The method of any one of claims 1 to 85, wherein:

- the first enzyme 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 intact stem-loop oligonucleotide by complementary base pairing to form a first double-stranded sequence comprising a portion of the second target,

hybridisation of a first primer oligonucleotide 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 sequence,

wherein the first polymerase comprising exonuclease activity digests the single-stranded loop portion of the first double-stranded sequence and thereby forms the split stem-loop oligonucleotide.

95. The method of any one of claims 1 to 85, wherein:

- the first enzyme is an exonuclease, and
- said treating the mixture comprises using conditions suitable for:

hybridisation of the second target to the single-stranded loop portion of the intact stem-loop oligonucleotide by complementary base pairing to form a first double-stranded sequence comprising a portion of the second target,

association of the first enzyme comprising exonuclease activity with the double-stranded sequence comprising the second target, and

catalytic activity of the first enzyme comprising exonuclease activity allowing it to digest the single-stranded loop portion of the first double-stranded sequence comprising the second target and thereby form the split stem-loop oligonucleotide.

96. The method of any one of claims 1 to 85, wherein:

- the first enzyme is a DNzyme or a ribozyme requiring a co-factor for catalytic activity, and said treating the mixture comprises using conditions suitable for:
 - binding of the cofactor to the first enzyme to render it catalytically active,
 - hybridisation of the DNzyme or ribozyme to the single-stranded loop portion of the intact stem-loop oligonucleotide by complementary base pairing,
 - catalytic activity of the DNzyme or ribozyme to digest the one or more unhybridised nucleotides of the single-stranded loop portion of the intact stem-loop oligonucleotide and thereby form the split stem-loop oligonucleotide,

wherein:

the second target is the co-factor.

97. The method of claim 96, wherein the co-factor is a metal ion, or a metal ion selected from: Mg^{2+} , Mn^{2+} , Ca^{2+} , Pb^{2+} .

98. The method of any one of claims 1 to 97, wherein:

- the first target differs from the second target; and/or

- the first oligonucleotide comprises or consists of a sequence that is not within the single-stranded loop portion of the intact stem-loop oligonucleotide.

99. The method of any one of claims 1 to 98, wherein:

- the first enzyme does not digest the second target.

100. The method of any one of claims 1 to 71, 74 to 91, or 94 to 99, wherein:

- any said enzyme does not digest the first target and/or the second target.

101. The method of any one of claim 1 to 100, 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.

102. The method of any one of claims 1 to 101, wherein said determining comprises detection of the first detectable signal and/or any said background signal(s):

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

103. The method of any one of claims 1 to 101, wherein said determining comprises detection of the first detectable signal and/or any said background signal(s):

- at one or more timepoints after said treating.

104. The method of any one of claims 1 to 101, wherein said determining comprises detection of the second detectable signal and/or any said background signal(s):

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

105. The method of any one of claims 1 to 101, wherein said determining comprises detection of the second detectable signal and/or any said background signal(s):

- one or more timepoints after said treating.

106. The method of any one of claims 1 to 105, wherein:

- said determining the presence or absence of the first and second targets comprises a melt curve analysis.

107. The method of claim 6, wherein:

- said determining the presence or absence of the first and second targets comprises a melt curve analysis comprising the first and second detectable signals and the optionally the first and second background signals.

108. The method of claim 55, wherein:

- said determining the presence or absence of the first and second targets comprises a melt curve analysis comprising the first and second detectable signals and the optionally the first and second background signals; or

- the first and second detectable signals and optionally the third background signal.

109. The method of any one of claims 1 to 108, wherein:

- the first target and/or the second target is an amplicon of a nucleic acid.

110. The method of any one of claims 1 to 109, wherein:

- the first target is a nucleic acid and/or the second target is a nucleic acid,
and

- the mixture further comprises reagents for amplification of said first and/or second target,

- said treating the mixture further comprises conditions suitable for conducting amplification of the first and/or second targets.

111. The method of claim 110, wherein:

- the amplification is any one or more 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/or reverse transcription polymerase chain reaction (RT-PCR).

112. The method of claim 110 or claim 111, 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.

113. The method of any one of claims 110 to 112, wherein said determining:

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

114. The method of any one of claims 110 to 113, wherein said determining occurs:

- at a first timepoint prior to said amplification; and
- at a second timepoint after completion of said amplification.

115. The method of any one of claims 110 to 114, wherein:

- the amplification method is polymerase chain reaction (PCR); and
- said determining occurs at multiple cycles optionally at each cycle.

116. The method of claim 110 or claim 111, further comprising normalising:

- the first detectable signal at the first temperature measured at a timepoint during or after said amplification using a positive control signal generated at the first temperature prior to said amplification and/or prior to said treating the reaction; and/or
- the second detectable signal at the second temperature measured at a timepoint during or after said amplification using a positive control signal generated at the second temperature prior to said amplification and/or prior to said treating the reaction.

117. The method of claim 110 or claim 111, further comprising normalising:

- the first detectable signal using a detectable signal generated by the intact stem-loop oligonucleotide at the first temperature prior to said amplification and/or prior to said treating the reaction; and/or

- the second detectable signal using a detectable signal generated by the intact stem-loop oligonucleotide at an additional temperature prior to said amplification and/or prior to said treating the reaction;

wherein the additional temperature is above the T_m of the intact stem-loop oligonucleotide.

118. The method of any one of claims 1 to 117 further comprising:

- generating a first target positive control signal using a known concentration of the first target and/or a known concentration of the first oligonucleotide after said modification.

119. The method of any one of claims 1 to 118:

- further comprising generating a first target positive control signal by repeating the method on a separate control sample comprising said first target.

120. The method of claim 119, wherein:

- the separate control sample comprising the first target comprises a known concentration of the first target.

121. The method of claim 119 or claim 120, wherein:

- the separate control sample comprising the first target further comprises the second target.

122. The method of any one of claims 1 to 121, further comprising:

- generating a second target positive control signal using a known concentration of the second target and/or a known concentration of the stem-loop oligonucleotide after said modification.

123. The method of any one of claims 1 to 122, further comprising:

- generating a second target positive control signal by repeating said method on a separate control sample comprising the second target.

124. The method of claim 123, wherein:

- the control sample comprising the second target comprises a known concentration of the second target.

125. The method of claim 123 or claim 124, wherein:

- said control sample comprising the second target further comprises said first target.

126. The method of any one of claims 1 to 125, further comprising:

- generating a combined positive control signal by repeating said method on a separate control sample comprising the first target and the second target.

127. The method of claim 126, wherein:

- the combined control sample comprises a known concentration of the first target and/or a known concentration of the second target.

128. The method of any one of claims 116 to 127, further comprising:

- normalising the first detectable signal and/or the second detectable signal using any said positive control signal.

129. The method of any one of claims 116 to 128, further comprising:

- assessing levels of a negative control signal by repeating the method of any one of claims 1 to 115 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.

130. The method of claim 129, further comprising:

- normalising the first detectable signal and/or the second detectable signal using said negative control signal.

131. The method of any one of claims 116 to 130, wherein:

- any said control signal is a fluorescent control signal.

132. The method of any one of claims 1 to 131, further comprising comparing the first and/or second detectable signals to a threshold value wherein:

- the threshold value is generated using detectable signals derived from a series of samples or derivatives thereof tested according to the method of any one of claims 1 to 115, 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.

133. The method of claim 132, wherein:

- the series of samples or derivatives thereof is tested using a known concentration of the first oligonucleotide and/or a known concentration of the intact stem-loop oligonucleotide.

134. The method of any one of claims 1 to 133, wherein:

- the sample is a biological sample obtained from a subject.

135. The method of any one of claims 1 to 133:

- wherein the method is performed *in vitro*.

136. The method of any one of claims 1 to 133:

- wherein the method is performed *ex vivo*.

137. The method of any one of claims 1 to 136, wherein:

- the first and second detectable moieties emit in the same colour region of the visible spectrum.

138. A composition comprising:

- a first oligonucleotide for detection of a first target, wherein the first target is a nucleic acid and complementary to at least a portion of the first oligonucleotide, and

- a first detection moiety, wherein:

the first detection moiety is capable of generating a first detectable signal upon modification of the first oligonucleotide, and

the modification is induced by hybridisation of the first target to the first oligonucleotide by complementary base pairing;

- an intact stem-loop oligonucleotide for detection of the second target, and 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, wherein at least one strand of the double-stranded stem portion comprises a second detection moiety; and

- a first enzyme capable of digesting one or more of the unhybridised nucleotides of the intact stem-loop oligonucleotide only when the second target is present in the sample, to thereby break the single-stranded loop portion and provide a split stem-loop oligonucleotide;

wherein:

- the second detection moiety is capable of generating a second detectable signal upon dissociation of the double-stranded stem portion of the split stem-loop oligonucleotide, and

- the first and second detection moieties are capable of generating detectable signals that cannot be differentiated at a single temperature using a single type of detector.

139. The composition of claim 138, wherein:

- the region of the first oligonucleotide which is complementary to the first target has a different melting temperature (T_m) to each strand of the double-stranded stem portion of the intact stem-loop oligonucleotide.

140. The composition of claim 138 or claim 139, wherein the first oligonucleotide differs in sequence from:

each strand of the double-stranded stem portion of the intact stem-loop oligonucleotide; and

the single-stranded loop portion of the intact stem-loop oligonucleotide.

141. The composition of any one of claims 138 to 140, wherein:

- the first oligonucleotide is a stem-loop oligonucleotide comprising a double-stranded stem portion of hybridised nucleotides on opposing strands of which are linked by an unbroken single-stranded loop portion of unhybridised nucleotides of which all or a portion, is/are complementary to the first target.

142. The composition of claim 141, wherein:

- the first target is hybridised to the first oligonucleotide by complementary base pairing causing dissociation of strands in the double-stranded stem portion of the first oligonucleotide thereby enabling the first detection moiety to provide the first detectable signal.

143. The composition of any one of claims 138 to 140, wherein:

- the first oligonucleotide is a stem-loop oligonucleotide comprising:
 - a double-stranded stem portion of hybridised nucleotides, opposing strands of which are linked by a single-stranded loop portion of unhybridised nucleotides, all or a portion of which is/are complementary to the first target, and
 - a second single-stranded portion extending from one of said opposing strands in a 3' direction and terminating with a sequence that is complementary to a portion of the first target, and
 - a blocker molecule preceding said sequence that is complementary to the portion of the first target.

144. The composition of claim 143, wherein:

- the first target is hybridised to the second single-stranded portion thereof by complementary base pairing;
 - the composition further comprises a polymerase capable of extending the second single-stranded portion using the first target as a template sequence to provide a double-stranded nucleic acid, wherein said blocker molecule is capable of preventing the polymerase extending the first target using said one opposing strand as a template, and
 - upon denaturing the double-stranded nucleic acid, the second single-stranded portion extended by the polymerase is capable of hybridising to the single-stranded loop portion of the first oligonucleotide by complementary base pairing to produce a signaling duplex and thereby enable the first detection moiety to provide a first detectable signal.

145. The composition of any one of claims 141 to 144, wherein:

- the first detection moiety is a fluorophore.

146. The composition of claim 145, wherein:

- the first oligonucleotide comprises a quencher molecule, and the fluorophore and the quencher molecule are located on opposing strands of the double-stranded stem portion of the first oligonucleotide.

147. The composition of any one of claims 138 to 140, wherein:

- the first oligonucleotide comprises:
 - a first double-stranded portion of hybridised nucleotides, a first strand of which extends into a single-stranded portion terminating with a complementary sequence capable of hybridising to a portion of the first target, wherein the first strand comprises a blocker molecule preceding said complementary sequence.
 - the composition further comprises a polymerase.

148. The composition of claim 147, wherein:

a portion of the first target is hybridised to said complementary sequence of the single-stranded portion by complementary base pairing; and

the composition further comprises a polymerase capable of extending the complementary sequence using the first target as a template sequence to provide a second double-stranded portion, wherein said blocker molecule prevents the polymerase extending the first target using the single-stranded portion as a template; and

when the first and second double-stranded portions are denatured, the complementary sequence extended by the polymerase is capable of hybridising to the first strand of the first double-stranded portion by complementary base pairing to produce a signaling duplex and thereby enable the first detection moiety to provide the first detectable signal.

149. The composition of claim 147 or claim 148, wherein:

- the first detection moiety is a fluorophore and the modification increases its distance from a quencher molecule;

150. The composition of claim 149, wherein:

- the first oligonucleotide comprises a quencher molecule, and the fluorophore and the quencher molecule are located on opposing strands of the first double-stranded portion.

151. The composition of any one of claims 138 to 140, wherein:

- the first oligonucleotide is complementary to a first portion of the target;
- the composition further comprises an additional oligonucleotide complementary to a second portion the first target, wherein the first and second portions of the first target flank one another but do not overlap, and are each capable of hybridising to the first target to form a duplex structure comprising:

(iii) a first double-stranded component by hybridising the first oligonucleotide to the target or by complementary base pairing, and

(iv) a second double-stranded component by hybridising the additional oligonucleotide to the target by complementary base pairing,

thereby bringing the first and additional oligonucleotides into proximity, and enabling the first detection moiety to provide the first detectable signal.

152. The composition of claim 151, wherein:

- the first detectable moiety is a fluorophore and the additional oligonucleotide comprises a quencher;
- said forming of the duplex structure further brings the fluorophore and quencher into proximity; and
- said detectable signal is a decrease in fluorescence provided by the first detection moiety.

153. The method of any one of claims 138 to 140, wherein:

- the first oligonucleotide is hybridised to the first target by complementary base pairing,

- the composition further comprises:

a primer hybridised to a portion of the first target by complementary base pairing, and

a polymerase with exonuclease activity capable of extending the primer using the first target as a template sequence to thereby digest the first oligonucleotide and modify the first oligonucleotide enabling the first detection moiety to provide the first detectable signal.

154. The composition of any one of claims 138 to 140, wherein:

- the first target is hybridised to the first oligonucleotide by complementary base pairing to thereby form a double-stranded duplex,

- the composition further comprises a restriction endonuclease capable of digesting a double-stranded duplex comprising the first target thereby modifying the first oligonucleotide and enable the first detection moiety to provide the first detectable signal.

155. The composition of claim 154, wherein:

- the restriction endonuclease is a nicking endonuclease capable of associating with and cleaving a strand of said double-stranded duplex, and said strand comprises the first oligonucleotide.

156. The composition of any one of claims 153 to 155, wherein:

- the first detection moiety is a fluorophore and said modifying of the first oligonucleotide increases the distance of the fluorophore from a quencher molecule.

157. The composition of claim 156, wherein:

- the first oligonucleotide comprises the quencher molecule.

158. A composition comprising:

- a first oligonucleotide for detection of a first target comprising a first detection moiety, wherein:

the first detection moiety is capable of generating a first detectable signal upon modification of the first oligonucleotide, and

the modification is induced by the first target;

- an intact stem-loop oligonucleotide for detection of the second target, and 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, wherein at least one strand of the double-stranded stem portion comprises a second detection moiety; and

- a first enzyme capable of digesting one or more of the unhybridised nucleotides of the intact stem-loop oligonucleotide only when the second target is present in the sample, to thereby break the single-stranded loop portion and provide a split stem-loop oligonucleotide;

wherein:

- the second detection moiety is capable of generating a second detectable signal upon dissociation of the double-stranded stem portion of the split stem-loop oligonucleotide, and

- the first and second detection moieties are capable of generating detectable signals that cannot be differentiated at a single temperature using a single type of detector.

159. The composition of claim 158, wherein the first oligonucleotide differs in sequence from:

each strand of the double-stranded stem portion of the intact stem-loop oligonucleotide; and

the single-stranded loop portion of the intact stem-loop oligonucleotide.

160. The composition of claim 158 or claim 159 wherein:

- the first target is a nucleic acid sequence;
- the composition further comprises:
 - a first primer complementary to a first sequence in the first target,
 - a second oligonucleotide comprising a component complementary to a second sequence in the first target that differs from the first sequence, and a tag portion that is not complementary to the first target,
 - a first polymerase comprising exonuclease activity, and
 - optionally a second polymerase.

161. The composition of claim 160, wherein:

- the first primer and the second oligonucleotide are each hybridised to the first target by complementary base pairing,
 - the first polymerase is capable of extending the first primer using the target as a template to thereby cleave off the tag portion, allowing the cleaved tag portion to hybridise to the first oligonucleotide by complementary base pairing, and
 - the first polymerase or the optional second polymerase is/are capable of extending the tag portion using the first oligonucleotide as a template to generate a double-stranded sequence comprising the first oligonucleotide thereby modify the first oligonucleotide and enabling the first detection moiety to provide the first detectable signal.

162. The composition of claim 160 or claim 161, wherein:

- the first oligonucleotide comprises a fluorophore and a quencher molecule.

163. The composition of claim 162, wherein:

- the first oligonucleotide comprises a fluorophore and a quencher molecule, and
- said extending the tag portion increases the distance between the fluorophore and the quencher molecule.

164. The composition of claim 158 or claim 159, wherein:

- the first target is a co-factor for enzyme catalytic activity;
- the composition further comprises a DNAzyme or a ribozyme requiring the co-factor for catalytic activity, and
- DNAzyme or ribozyme is capable of binding to the first target and hybridising to the first oligonucleotide by complementary base pairing, thereby digesting and modifying the first oligonucleotide enabling the first detection moiety to generate the first detectable signal.

165. The composition of claim 164, wherein the co-factor is a metal ion, or a metal ion selected from: Mg^{2+} , Mn^{2+} , Ca^{2+} , Pb^{2+} .

166. The method of claim 158 or claim 159, wherein:

- the first oligonucleotide is a substrate for a multi-component nucleic acid enzyme (MNAzyme);
- the composition further comprises an MNAzyme capable of cleaving the first oligonucleotide when the first target is present in the sample; and
- wherein the MNAzyme is capable of binding to the first target and hybridising to the first oligonucleotide by complementary base pairing via its substrate arms, and said hybridisation facilitates cleavage of the first oligonucleotide thereby modifying it and enabling the first detection moiety to provide the first detectable signal.

167. The composition of claim 166, wherein:

- the first target is a nucleic acid sequence; and
- the first target is hybridised to the sensor arms of the MNAzyme by complementary base pairing to thereby facilitate assembly of the MNAzyme.

168. The composition of claim 158 or claim 159, wherein:

- the first target is an analyte, protein, compound or molecule;
- the first oligonucleotide is a substrate for an aptazyme; and
- the composition further comprises an aptazyme comprising an aptamer portion capable of binding to the first target, and a nucleic acid enzyme portion capable of digesting the first oligonucleotide and thereby modifying it enabling the first detection moiety to provide the first detectable signal.

169. The composition of claim 168, wherein:

- the first target is bound to the aptamer portion of the aptazyme and the first oligonucleotide is hybridised to the active nucleic acid enzyme portion by complementary base pairing facilitating digestion of the first oligonucleotide and thereby modifying it enabling the first detection moiety to provide the first detectable signal.

170. The composition of any one of claims 166 to 169, wherein:

- the first detection moiety is a fluorophore and said modifying the first oligonucleotide increases the distance of the fluorophore from a quencher molecule.

171. The composition of claim 170, wherein:

- the first oligonucleotide comprises the quencher molecule.

172. The composition of any one of claims 138 to 144, 147, 148, 151, 153 to 155, 158 to 161, and 164 to 169, wherein:

- the first detection moiety is: a nanoparticle, a metallic nanoparticle, a noble metal nanoparticle, an alkali metal nanoparticle, a gold nanoparticle, or a silver nanoparticle; to which the first oligonucleotide is bound; and
- the first detectable signal is:
 - (i) a change in refractive index,
 - (ii) a change in colour; and/or
 - (iii) a change in absorption spectrum,arising from the first detection moiety following said modification of the first oligonucleotide.

173. The composition of claim 172, wherein:

- the first detection moiety is an electrochemical agent to which the first oligonucleotide is bound;

- the first detectable signal is a change in electrochemical signal.

174. The composition of claim 173, wherein:

- the electrochemical agent is selected from any one or more of a nanoparticle, Methylene blue, Toluene blue, Oracet Blue, Hoechst 33258, [Ru(phen)₃]²⁺, ferrocene, and/or daunomycin.

175. The composition of any one of claims 172 to 174, wherein:

- the second detection moiety is: a nanoparticle, a metallic nanoparticle, a noble metal nanoparticle, an alkali metal nanoparticle, a gold nanoparticle, or a silver nanoparticle; to which at least one strand of the double-stranded stem portion of the second oligonucleotide is bound and

- the second detectable signal is:

- (i) a change in refractive index,
- (ii) a change in colour; and/or
- (iii) a change in absorption spectrum,

arising upon said dissociation of the double-stranded stem portion of the split stem-loop oligonucleotide.

176. The composition any one of claims 172 to 174, wherein:

- the second detection moiety is an electrochemical agent to which the second oligonucleotide is bound; and

- the second detectable signal is a change in electrochemical signal arising upon said dissociation of the double-stranded stem portion of the split stem-loop oligonucleotide.

177. The composition of claim 176, wherein:

- the electrochemical agent is selected from any one or more of a nanoparticle, Methylene blue, Toluene blue, Oracet Blue, Hoechst 33258, [Ru(phen)₃]²⁺, ferrocene, and/or daunomycin.

178. The composition of any one of claims 145, 146, 149, 150, 152, 156, 157, 162, 163, 170, and 171 wherein:

- the second detection moiety is a fluorophore, and

- the second detectable signal provided by said second detection moiety upon dissociation of the double-stranded stem portion of the split stem-loop oligonucleotide increases the distance of the fluorophore from a quencher molecule.

179. The composition of claim 178, wherein:

- the fluorophore and quencher molecule are located on opposing strands of the double-stranded stem portion of the stem-loop oligonucleotide.

180. The composition of any one of claims 138 to 179, wherein:

- the first enzyme is a first MNAzyme,
- the first MNAzyme is bound to the second target,
- the substrate arms of said first MNAzyme are hybridised by complementary base pairing to the single loop portion of the intact stem-loop oligonucleotide, thereby facilitating digestion of the one or more unhybridised nucleotides of the intact stem-loop oligonucleotide and providing the split stem-loop oligonucleotide.

181. The composition of claim 180, wherein:

- the second target or is a nucleic acid sequence; and
- the second target is hybridised to the sensor arms of the first MNAzyme by complementary base pairing to thereby facilitate assembly of the first MNAzyme.

182. The composition of any one of any one of claims 138 to 179, wherein:

- the second target is an analyte, protein, compound or molecule;
- the first enzyme is an aptazyme comprising an aptamer capable of binding to the second target; and
- the aptamer is bound to the second target thereby rendering the first enzyme catalytically active.

183. The composition of claim 182, wherein:

- the first enzyme is any one of an: apta-DNAzyme, apta-ribozyme, apta-MNAzyme.

184. The composition of any one of claims 138 to 179, wherein:

- the second target is an analyte, protein, compound or molecule;

- the single-stranded loop portion of the intact stem-loop oligonucleotide is a substrate for an aptazyme; and
- the composition further comprises an aptazyme comprising an aptamer portion capable of binding to the second target, and a nucleic acid enzyme portion capable of digesting the one or more unhybridised nucleotides of the intact stem-loop oligonucleotide to thereby form the split stem-loop oligonucleotide.

185. The composition of claim 184, wherein:

- the second target is bound to the aptamer portion of the aptazyme and the single-stranded loop portion of the intact stem-loop oligonucleotide is hybridised to the active nucleic acid enzyme portion by complementary base pairing, facilitating digestion of the one or more unhybridised nucleotides of the intact stem-loop oligonucleotide to thereby form the split stem-loop oligonucleotide.

186. The composition of any one of claims 138 to 179, wherein:

- the second target is a nucleic acid sequence; and
- the first enzyme is a first restriction endonuclease, and
- the second target is hybridised to the single-stranded loop portion of the intact stem-loop oligonucleotide by complementary base pairing to form a double-stranded sequence for the first restriction endonuclease to associate with and digest the one or more unhybridised nucleotides of the intact stem-loop oligonucleotide to thereby form the split stem-loop oligonucleotide.

187. The composition of claim 186, wherein:

- the first restriction endonuclease is a first nicking endonuclease capable of associating with and cleaving a strand of said double-stranded sequence for the first restriction endonuclease, and said strand comprises the intact stem-loop oligonucleotide.

188. The composition of any one of claims 138 to 179, wherein:

- the first enzyme comprises a polymerase with exonuclease activity,
- the second target is hybridised to the single-stranded loop portion of the intact stem-loop oligonucleotide by complementary base pairing to form a first double-stranded sequence comprising a portion of the second target,
- the composition further comprises a first primer oligonucleotide hybridised by complementary base pairing 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, and

- the primer can be extended using the polymerase with exonuclease activity and the second target as a template sequence, digesting the single-stranded loop portion of the first double stranded sequence and thereby forming a split stem-loop oligonucleotide.

189. The composition of any one of claims 138 to 179, wherein:

- the first enzyme is an exonuclease, and
- the second target is hybridised by complementary base pairing to the single-stranded loop portion of the intact stem-loop oligonucleotide forming a first double-stranded sequence comprising a portion of the second target, to which the first enzyme comprising exonuclease activity can associate and thereby digest the single-stranded loop portion of the first double stranded sequence comprising the second target to form the split stem-loop oligonucleotide.

190. The composition of any one of claims 138 to 179, wherein:

- the first enzyme is a DNAzyme or a ribozyme requiring a co-factor for catalytic activity, and
- the second target is the co-factor and is bound to the DNAzyme or ribozyme,
- the DNAzyme or ribozyme is hybridised to the single-stranded loop portion of the intact stem-loop oligonucleotide by complementary base pairing, allowing it to digest the one or more unhybridised nucleotides of the single-stranded loop portion of the intact stem-loop oligonucleotide and thereby form the split stem-loop oligonucleotide.

191. The composition of claim 190, wherein the co-factor is a metal ion, or a metal ion selected from: Mg^{2+} , Mn^{2+} , Ca^{2+} , Pb^{2+} .

192. The composition of any one of claims 138 to 150, 153, 156 to 158, 166 or 167, wherein:

- the first oligonucleotide is selected from any one or more of: a Molecular Beacon®, a Scorpions® primer, a TaqMan® primer, or an MNzyme substrate.

193. The composition of any one of claims 138 to 192 wherein:

- the first target and/or the second target is an amplicon of a nucleic acid.