

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 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; and optionally 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.

4. The method of claim 3, 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;
 - 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; and
 - 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,
- and optionally wherein:
- (a)
- 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, or

(b)

- 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, or

(c)

- 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, or

(d)

- 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.

5. The method of claim 3, 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,
- and optionally wherein:
- (a)
- 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, or

(b)

- 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, or

(c)

- 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, or
- (d)
- 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.

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

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

7. The method of claim 3, 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,
 - and optionally wherein:
 - (a)
 - 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, or

(b)

- 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, or

(c)

- 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, or
- (d)
 - 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.

8. The method of claim 3, 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,

and optionally wherein:

(a)

- 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, or

(b)

- 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, or

(c)

- 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, or
- (d)
- 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.

9. The method of claim 3, 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:
 - (i) a first double-stranded component by hybridising the first oligonucleotide to the target by complementary base pairing, and

(ii) 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, optionally 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, and optionally 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.

10. The method of any one of claims 1 to 3, wherein:

(a)

- 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, or

(b)

- 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.

11. The method of claim 1 or claim 2, 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; optionally 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.

12. The method of claim 11, wherein:

(a)

- 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, or

(b)

- 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, or

(c)

- 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.

13. The method of claim 11 or claim 12 wherein:

(a)

- 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, or

(b)

- 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.

14. The method of any one of claims 11 to 13, wherein:

(a)

- 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 MNAzyme to the first target and hybridisation of the substrate arms of the MNAzyme 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, or

(b)

- the first oligonucleotide is a substrate for an aptazyme;

- the first target is an analyte, protein, compound or molecule;
- the mixture further comprises an aptazyme comprising an aptamer capable of binding to the first target; and

- said treating the mixture further comprises:
 - binding of the aptazyme to the first target and 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, or

(c)

- 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, or

(d)

- 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, or

- (e)
- the mixture further comprises a DNzyme 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 DNzyme or ribozyme to render it catalytically active,
 - hybridisation of the DNzyme or ribozyme to the first oligonucleotide by complementary base pairing, and
 - catalytic activity of the DNzyme 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.

15. The method of any one of claims 1 to 14, wherein:

- the first enzyme is a first MNzyme, and
- said treating the mixture comprises:
 - binding of the first MNzyme to the second target and hybridisation of substrate arms of said first MNzyme 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, and 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 MNzyme by complementary base pairing to thereby facilitate assembly of the first MNzyme.

16. The method of any one of claims 1 to 14, wherein:

- (a)
- 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, or

(b)

- 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, or

(c)

- 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, or

(d)

- the first enzyme is a DNAzyme 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 DNAzyme or ribozyme to the single-stranded loop portion of the intact stem-loop oligonucleotide by complementary base pairing,
 - catalytic activity of the DNAzyme 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.

17. The method of any one of claims 1 to 16, wherein:

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

18. The method of any one of claims 1 to 17 further comprising:

- generating a first target positive control signal using a known concentration of the first target and a known concentration of the first oligonucleotide after said modification, and/or generating a first target positive control signal by repeating the method on a separate control sample comprising said first target; and/or

- generating a second target positive control signal using a known concentration of the second target and a known concentration of the stem-loop oligonucleotide after said modification, and/or generating a second target positive control signal by repeating said method on a separate control sample comprising the second target; and/or

- generating a combined positive control signal by repeating said method on a separate control sample comprising the first target and the second target, optionally wherein the combined control sample comprises a known concentration of the first target and/or a known concentration of the second target.

19. The method of claim 18, further comprising:

(a)

- assessing levels of a negative control signal by repeating the method of any one of claims 1 to 17 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; and

further comprising normalising the first detectable signal and/or the second detectable signal using said negative control signal, and/or

(b)

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 17, 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, optionally 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.

20. The method of any one of claims 1 to 19, wherein:

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

21. 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.