~~WHAT IS~~ CLAIM~~SED~~:

~~1. A method for repeated signal detection with a reporter-labeled hairpin, the method comprising:~~

~~a) providing a sample possibly containing up to N targets as well as possibly other molecules that are not targets;~~

~~b) providing N probe sets (each targeting one of N target types) each comprising either: i) one or more HCR initiator-labeled probes, or ii) one or more probe units each comprising two or more HCR fractional initiator probes;~~

~~c) optionally washing the sample;~~

~~d) providing M HCR amplifiers (for M ≤ N; each labeled with a distinct reporter) corresponding to M of the N probe sets;~~

~~e) optionally washing the sample;~~

~~f) detecting M signals corresponding to the M reporters;~~

~~g) removing the M signals from the sample; and~~

~~h) optionally repeating one or more of steps b-g until signal detection has been performed for all N targets;~~

~~wherein a probe set comprises either:~~

~~a) one or more HCR initiator-labeled probes, or~~

~~b) one or more probe units,~~

~~wherein an HCR initiator-labeled probe comprises:~~

~~one or more target-binding regions and~~

~~one or more initiators,~~

~~wherein a probe unit comprises: two or more HCR fractional initiator probes,~~

~~wherein an HCR fractional initiator probe comprises:~~

~~a target-binding region and~~

~~a fractional initiator, and~~

~~wherein an HCR amplifier comprises two or more HCR hairpins,~~

~~wherein an HCR hairpin comprises an input domain comprising:~~

~~a single-stranded toehold; and~~

~~a stem section,~~

~~wherein an HCR hairpin further comprises an output domain comprising:~~

a single-stranded loop; and

a complement to the stem section, and

wherein an HCR hairpin further comprises a reporter.

2. A method for repeated signal detection with a reporter-labeled hairpin, the method comprising:

a) providing a sample possibly containing one or more targets as well as possibly other molecules that are not targets;

b) providing one or more probe sets each comprising either: i) one or more HCR initiator-labeled probes, or ii) one or more probe units each comprising two or more HCR fractional initiator probes;

c) optionally washing the sample;

d) providing one or more HCR amplifiers (each labeled with one or more reporters);

e) optionally washing the sample;

f) detecting one or more signals from one or more reporters;

g) optionally removing one or more probe sets from the sample;

h) optionally removing one or more HCR amplifiers from the sample;

i) optionally removing one or more reporters from the sample; and

j) optionally removing one or more signals from the sample;

wherein a probe set comprises either:

a) one or more HCR initiator-labeled probes, or

b) one or more probe units,

wherein an HCR initiator-labeled probe comprises:

one or more target-binding regions and

one or more initiators,

wherein a probe unit comprises two or more HCR fractional initiator probes,

wherein an HCR fractional initiator probe comprises:

a target-binding region and

a fractional initiator;

wherein an HCR amplifier comprises two or more HCR hairpins;

wherein an HCR hairpin comprises: an input domain comprising:

a single-stranded toehold and

a stem section,

wherein an HCR hairpin further comprises an output domain comprising:

a single-stranded loop and

a complement to the stem section, and

wherein an HCR hairpin further comprises one or more reporters.

3. A method of repeated signal detection with substrate-labeled hairpins, the method comprising:

a) providing a sample possibly containing up to N targets as well as possibly other molecules that are not targets;

b) providing N probe sets each comprising either: i) one or more HCR initiator-labeled probes, or ii) one or more probe units each comprising two or more HCR fractional initiator probes;

c) optionally washing the sample;

d) providing N HCR amplifiers (each labeled with a distinct substrate) corresponding to the N probe sets;

e) optionally washing the sample;

f) providing M label probes (for $M \leq N$; each conjugated to a distinct reporter) corresponding to M of the N distinct substrates;

g) optionally washing the sample;

h) detecting M signals corresponding to the M distinct reporters;

i) removing the M signals from the sample; and

j) optionally repeating one or more of steps f-i until signal detection has been performed for all N targets;

wherein a probe set comprises either:

a) one or more HCR initiator-labeled probes, or

b) one or more probe units;

wherein an HCR initiator-labeled probe comprises:

one or more target-binding regions and

one or more initiators,

wherein a probe unit comprises: two or more HCR fractional initiator probes;

wherein an HCR fractional initiator probe comprises:

a target-binding region and

a fractional initiator;

wherein an HCR amplifier comprises two or more HCR hairpins;

wherein an HCR hairpin comprises an input domain comprising:

a single-stranded toehold and

a stem section;

wherein an HCR hairpin further comprises an output domain comprising:

a single-stranded loop and

a complement to the stem section;

wherein an HCR hairpin further comprises a substrate, and

wherein a label probe comprises:

a substrate-binding region and

a reporter.

4. A method of repeated signal detection with substrate-labeled hairpins, the method comprising:

a) providing a sample possibly containing one or more targets as well as possibly other molecules that are not targets;

b) providing one or more probe sets each comprising either: i) one or more HCR initiator-labeled probes, or ii) one or more probe units each comprising two or more HCR fractional initiator probes;

c) optionally washing the sample;

d) providing one or more HCR amplifiers (each labeled with a substrate) corresponding to one or more probe sets;

e) optionally washing the sample;

f) providing one or more label probes (each conjugated to a reporter) corresponding to one or more substrates;

g) optionally washing the sample;

h) detecting one or more signals corresponding to one or more reporters;

i) removing one or more signals from the sample; and

j) optionally repeating any of steps b-i one or more times in any order;

wherein a probe set comprises either:

a) one or more HCR initiator-labeled probes, or

b) one or more probe units,

wherein an HCR initiator-labeled probe comprises:

one or more target-binding regions and

one or more initiators,

wherein a probe unit comprises two or more HCR fractional initiator probes,

wherein an HCR fractional initiator probe comprises:

a target-binding region and

a fractional initiator,

wherein an HCR amplifier comprises two or more HCR hairpins,

wherein an HCR hairpin comprises an input domain comprising:

a single-stranded toehold and

a stem section,

wherein an HCR hairpin further comprises an output domain comprising:

a single-stranded loop and

a complement to the stem section,

wherein an HCR hairpin further comprises a substrate, and

wherein a label probe comprises:

a substrate-binding region and

a reporter.

5. A method of repeated signal detection with reporter and/or substrate-labeled hairpins, the method comprising:

a) providing a sample possibly containing one or more targets as well as possibly other molecules that are not targets;

b) providing one or more HCR probe sets each comprising either: i) one or more HCR initiator-labeled probes, or ii) one or more probe units each comprising two or more HCR fractional initiator probes;

c) providing one or more HCR amplifiers (each labeled with one or more reporters and/or one or more substrates) corresponding to one or more probe sets;

d) optionally providing one or more label probes (each conjugated to one or more reporters) corresponding to one or more substrates;

e) detecting one or more signals;

f) optionally washing the sample;

g) optionally removing one or more signals from the sample;

h) optionally removing one or more reporters from the sample;

i) optionally removing one or more label probes from the sample;

j) optionally removing one or more HCR amplifiers from the sample;

k) optionally removing one or more probe sets from the sample; and

l) optionally repeating any of the above steps in any order;

wherein a probe set comprises either:

a) one or more HCR initiator-labeled probes, or

b) one or more probe units,

wherein an HCR initiator-labeled probe comprises:

one or more target-binding regions and

one or more initiators,

wherein a probe unit comprises two or more HCR fractional initiator probes,

wherein an HCR fractional initiator probe comprises:

a target-binding region and

a fractional initiator,

wherein an HCR amplifier comprises two or more HCR hairpins,

wherein the HCR hairpin comprises an input domain comprising:

a single-stranded toehold and

a stem section,

wherein an HCR hairpin further comprises an output domain comprising:

a single-stranded loop and

a complement to the stem section,

wherein an HCR hairpin further comprises:

one or more reporters and/or

one or more substrates, and

wherein a label probe comprises:

a substrate-binding region and

one or more reporters.

6. A method of repeated signal detection with reporter and/or substrate-labeled hairpins, the method comprising:

a) providing a sample possibly containing one or more targets as well as possibly other molecules that are not targets;

b) performing any of steps c-g one or more times in any order;

c) providing one or more HCR probe sets each comprising either: i) one or more HCR initiator-labeled probes, or ii) one or more probe units each comprising two or more HCR fractional initiator probes;

d) providing one or more HCR amplifiers that directly or indirectly generate one or more signals;

e) optionally washing the sample;

f) detecting one or more signals; and

g) optionally removing one or more signals;

wherein a probe set comprises either:

a) one or more HCR initiator-labeled probes, or

b) one or more probe units;

wherein an HCR initiator-labeled probe comprises:

one or more target-binding regions and

one or more initiators,

wherein a probe unit comprises two or more HCR fractional initiator probes,

wherein an HCR fractional initiator probe comprises:

a target-binding region and

a fractional initiator,

wherein an HCR amplifier comprises two or more HCR hairpins,

wherein an HCR hairpin comprises an input domain comprising:

a single-stranded toehold and

a stem section,

wherein an HCR hairpin further comprises an output domain comprising:

a single-stranded loop and

a complement to the stem section, and

wherein an HCR hairpin further comprises:

one or more reporters and/or

one or more substrates.

7. A method of HCR involving overlapping binding sites, the method comprising:

a) providing a sample possibly containing a target as well as possibly other molecules that are not targets;

b) providing a probe set comprising one or more probe units each comprising two or more HCR fractional initiator probes where the target-binding regions on the probes within each probe unit are configured to bind to overlapping binding sites on the target;

c) optionally washing the sample;

d) providing an HCR amplifier labeled with a reporter and/or a substrate;

e) optionally washing the sample;

f) optionally providing a label probe (conjugated to a reporter) corresponding to the substrate;

g) optionally washing the sample; and

h) detecting a signal from the reporter.

wherein a probe set comprises one or more probe units,

wherein a probe unit comprises two or more HCR fractional initiator probes,

wherein an HCR fractional initiator probe comprises:

a target-binding region and

a fractional initiator,

wherein the target-binding regions on the probes within each probe unit are configured to bind to overlapping binding sites on the target,

wherein an HCR amplifier comprises two or more HCR hairpins,

wherein an HCR hairpin comprises an input domain comprising:

a single-stranded toehold and

a stem section,

wherein an HCR hairpin further comprises an output domain comprising:

a single-stranded loop and

a complement to the stem section,

wherein an HCR hairpin further comprises:

a reporter and/or

a substrate;

wherein a label probe comprises:

a substrate-binding region and

a reporter.

8. A method of HCR involving overlapping binding sites, the method comprising:

a) providing a sample possibly containing a target as well as possibly other molecules that are not targets

b) providing a probe set comprising one or more probe units each comprising two or more HCR fractional initiator probes where the fractional initiators on the probes within each probe unit are configured to bind to overlapping binding sites on an HCR hairpin;

c) optionally washing the sample;

d) providing an HCR amplifier labeled with a reporter and/or a substrate;

e) optionally washing the sample;

f) optionally providing a label probe (conjugated to a reporter) corresponding to the substrate;

g) optionally washing the sample; and

h) detecting a signal from the reporter.

wherein a probe set comprises one or more probe units;

wherein a probe unit comprises two or more HCR fractional initiator probes,

wherein an HCR fractional initiator probe comprises:

a target-binding region and

a fractional initiator,

wherein the fractional initiators on the probes within each probe unit are configured to bind to overlapping binding sites on an HCR hairpin,

wherein an HCR amplifier comprises two or more HCR hairpins,

wherein an HCR hairpin comprises an input domain comprising:

a single-stranded toehold and

a stem section,

wherein an HCR hairpin further comprises an output domain comprising:

a single-stranded loop and

a complement to the stem section,

wherein an HCR hairpin further comprises:

a reporter and/or

a substrate, and

wherein a label probe comprises:

a substrate-binding region and

a reporter.

9. A method of HCR involving overlapping binding sites with repeated signal detection, the method comprising:

a) providing a sample possibly containing one or more targets as well as possibly other molecules that are not targets

b) providing one or more probe sets each comprising either: i) one or more HCR initiator-labeled probes, or ii) one or more probe units each comprising two or more HCR fractional initiator probes where target-binding regions on the probes within each probe unit are configured to bind to overlapping or non-overlapping binding sites on a target and where fractional initiators on the probes within each probe unit are configured to bind to overlapping or non-overlapping binding sites on an HCR hairpin;

c) optionally washing the sample;

d) providing one or more HCR amplifiers each labeled with one or more reporters and/or substrates;

e) optionally washing the sample;

f) optionally providing one or more label probes (each conjugated to one or more reporters) corresponding to one or more substrates;

g) optionally washing the sample;

h) detecting a signal from one or more reporters;

i) optionally removing one or more signals from the sample;

j) optionally removing one or more reporters from the sample;

k) optionally removing one or more label probes from the sample;

l) optionally removing one or more amplifiers from the sample;

m) optionally removing one or more probe sets from the sample; and

n) optionally repeating any of the above steps in any order;

wherein a probe set comprises either:

a) one or more HCR initiator-labeled probes, or

b) one or more probe units,

wherein an HCR initiator-labeled probe comprises:

one or more target-binding regions and

one or more initiators,

wherein a probe unit comprises two or more HCR fractional initiator probes,

wherein an HCR fractional initiator probe comprises:

a target-binding region and

a fractional initiator,

wherein the target-binding regions within a probe unit are configured to bind to overlapping or non-overlapping binding sites on the target,

wherein the fractional initiators on the probes within each probe unit are configured to bind to overlapping or non-overlapping binding sites on an HCR hairpin,

wherein an HCR amplifier comprises two or more HCR hairpins,

wherein an HCR hairpin comprises an input domain comprising:

a single-stranded toehold and

a stem section,

wherein an HCR hairpin further comprises an output domain comprising:

a single-stranded loop and

a complement to the stem section,

wherein an HCR hairpin further comprises:

one or more reporters and/or

one or more substrates,

wherein a label probe comprises:

a substrate-binding region and

one or more reporters.

10. The method of any one of claims 1-9, wherein the target is a nucleic acid sequence.

11. The method of any one of claims 1 or 3, wherein N is 1 to 1000.

12. The method of claims 1 or 3, wherein M is 2-10,000

13. The method of any one of claims 1-12, wherein the substrate is selected from the group consisting of a hapten, digoxigenin (DIG), dinitrophenyl (DNP), biotin, a fluorophore, a nucleic acid domain comprising 4-50 nucleotides, a substrate that recruits an enzyme that catalyzes deposition of reporter molecules, a fractional substrate, a nucleic acid domain that directly or indirectly mediates localization of reporters.

14. The method of any one of claims 1-13, wherein the reporter is a fluorophore, a chromophore, a luminophore, a phosphor, a FRET pair, a member of a FRET pair, a quencher, a fluorophore/quencher pair, a rare-earth element or compound, a radioactive molecule, a magnetic molecule.

15. The method of any one of claims 1-14, further comprising fixing the sample.

16. The method of any one of claims 1-15, further comprising permeabilizing the sample.

17. The method of any one of claims 1-16 further comprising removing one or more signals from the sample.

18. The method of any one of claims 1-17, further comprising removing one or more reporters from the sample.

19. The method of any one of claims 1-18 further comprising removing one or more label probes from the sample.

20. The method of any one of claims 1-19 further comprising removing one or more amplifiers from the sample.

21. The method of any one of claims 1-20 further comprising removing one or more probe sets from the sample.

22. The method of any one of claims 1-21 further comprising repeating any of the above steps in any order.

23. The method of any one of claims 1-21, wherein the method is conducted in alphabetical order as lettered in the claim.

~~24. The method of any one of claims 1-23, wherein the probe units each comprise two or more HCR fractional initiator probes, and wherein an HCR fractional initiator probe comprises a target-binding region and a fractional initiator.~~

~~25. The method of any one of claims 1-24, wherein overlapping binding is involved and wherein the overlap is at least 1, 2, or 3 nucleotides.~~

~~26~~1. A method, comprising:

providing:

a first fractional initiator probe comprising a first fractional initiator;

a second fractional initiator probe comprising a second fractional initiator;

a first hairpin monomer, comprising:

a first input domain, comprising a first toehold and a first stem section,

a first output domain, comprising a first hairpin loop and a complement to the first stem section, and

a first hapten molecule;

a second hairpin monomer, comprising:

a second input domain, comprising a second toehold and a second stem section,

a second output domain, comprising a second hairpin loop and a complement to the second stem section, and

a second hapten molecule;

a target molecule; and

incubating the first fractional initiator probe and the second fractional initiator probe with the target.

~~27~~2. The method of claim ~~26~~1, wherein the incubating binds the first fractional initiator probe to the target molecule and binds the second fractional initiator probe to the target molecule.

~~28~~3. The method of ~~any one of~~ claim~~s 26-27~~1 or 2, further comprising:

binding the first hairpin monomer to both of the first fractional initiator and the second fractional initiator;

binding the second hairpin monomer to the first hairpin monomer;

providing an anti-hapten molecule labeled with one or more reporter entities, wherein the reporter entity is an enzyme that mediates CARD;

providing one or more CARD-substrates;

measuring a signal from one or more deposited reporters generated from the CARD-substrate by the enzyme that mediates CARD.

~~294~~. The method of any one of claims ~~261~~-~~283~~, wherein the anti-hapten molecule is an anti-hapten antibody or an anti-hapten nanobody.

~~305~~. The method of any one of claims ~~261~~-~~294~~, wherein the at least one target is a nucleic acid or a protein.

~~316~~. The method of any one of claims ~~261~~-~~305~~, wherein the at least one target molecule is an RNA.

~~327~~. A method, comprising:

providing:

at least one initiator-labeled probe comprising at least one initiator;

a first hairpin monomer, comprising:

a first input domain, comprising a first toehold and a first stem section,

a first output domain, comprising a first hairpin loop and a complement to the first stem section, and

a first hapten molecule;

a second hairpin monomer, comprising:

a second input domain, comprising a second toehold and a second stem section,

a second output domain, comprising a second hairpin loop and a complement to the second stem section, and

a second hapten molecule,

a target molecule; and

incubating the at least one initiator-labeled probe comprising at least one initiator with the target.

~~33~~8. The method of claim ~~32~~7, wherein the incubating binds the at least one initiator-labeled probe comprising at least one initiator to the target molecule.

~~34~~9. The method of ~~any one of~~ claim~~s~~ ~~32~~7-~~33~~ or 8, further comprising:

binding the first hairpin monomer to the at least one initiator;

binding the second hairpin monomer to the first hairpin monomer;

providing an anti-hapten molecule labeled with one or more reporter entities, wherein the reporter entity is an enzyme that mediates CARD;

providing one or more CARD-substrates;

measuring a signal from one or more deposited reporters generated from the CARD-substrate by the enzyme that mediates CARD.

~~35~~10. The method of any one of claims ~~32~~7-~~34~~9, wherein the target molecule is a protein or a nucleic acid.

~~36~~11. The method of any one of claims ~~32~~7-~~35~~10, wherein the anti-hapten molecule is an anti-hapten antibody or an anti-hapten nanobody.

~~37~~12. The method of claim ~~36~~11, wherein the anti-hapten antibody is a primary antibody.

1~~3~~8. The method of claim ~~36~~11, wherein the anti-hapten antibody comprises a primary antibody that binds the hapten and the method further comprises a secondary antibody (labeled with one or more reporter entities) that binds the primary antibody.

~~39. A method, comprising:~~

~~providing:~~

~~a first fractional initiator probe comprising a first fractional initiator;~~

~~a second fractional initiator probe comprising a second fractional initiator;~~

~~a first hairpin monomer, comprising:~~

~~a first input domain, comprising a first toehold and a first stem section,~~

~~a first output domain, comprising a first hairpin loop and a complement to the first stem section, and~~

~~a substrate;~~

~~a second hairpin monomer, comprising:~~

a second input domain, comprising a second toehold and a second stem section,

a second output domain, comprising a second hairpin loop and a complement to the second stem section, and

the substrate,

a target molecule; and

incubating the first fractional initiator probe and the second fractional initiator probe with the target.

40. The method of claim 39, wherein the incubating binds the first fractional initiator probe to the target molecule and binds the second fractional initiator probe to the target molecule.

41. The method of any one of claims 39-40, further comprising:

binding the first hairpin monomer to both of the first fractional initiator and the second fractional initiator;

binding the second hairpin monomer to the first hairpin monomer;

providing a substrate-binding region labeled with one or more reporter entities, wherein the substrate-binding region binds to the substrate, and wherein the reporter entity is an enzyme that mediates CARD;

providing one or more CARD-substrates;

measuring a signal from one or more deposited reporters generated from the CARD-substrate by the enzyme that mediates CARD.

42. The method of any one of claims 39-41, wherein the at least one target is a nucleic acid.

43. The method of any one of claims 39-42, wherein the at least one target molecule is an RNA.

44. A method, comprising:

providing:

a first fractional initiator probe comprising a first fractional initiator;

a second fractional initiator probe comprising a second fractional initiator;

a first hairpin monomer, comprising:

~~a first input domain, comprising a first toehold and a first stem section,~~

~~a first output domain, comprising a first hairpin loop and a complement to the first stem section, and~~

~~a first fractional substrate;~~

~~a second hairpin monomer, comprising:~~

~~a second input domain, comprising a second toehold and a second stem section,~~

~~a second output domain, comprising a second hairpin loop and a complement to the second stem section, and~~

~~a second fractional substrate,~~

~~a target molecule; and~~

~~incubating the first fractional initiator probe and the second fractional initiator probe with the target molecule.~~

~~45. The method of claim 44, wherein the incubating binds the first fractional initiator probe to the target molecule and binds the second fractional initiator probe to the target molecule.~~

~~46. The method of any one of claims 44-45, further comprising:~~

~~binding the first hairpin monomer to both of the first fractional initiator and the second fractional initiator;~~

~~binding the second hairpin monomer to the first hairpin monomer;~~

~~obtaining a full substrate comprising the first and second fractional substrate;~~

~~providing a substrate-binding region labeled with one or more reporter entities, wherein the substrate-binding region binds to the full substrate, and wherein the reporter entity is an enzyme that mediates CARD;~~

~~providing one or more CARD-substrates;~~

~~measuring a signal from one or more deposited reporters generated from the CARD-substrate by the enzyme that mediates CARD.~~

~~47. The method of any one of claims 44-46, wherein the at least one target is a nucleic acid.~~

~~48. The method of any one of claims 44-47, wherein the at least one target molecule is an RNA.~~

14. The method of claim 1, wherein the probes are secondary probes and wherein the target is a secondary target, and wherein the secondary target is a primary probe that binds a primary target.

15. The method of claim 7, wherein the probes are secondary probes and wherein the target is a secondary target, and wherein the secondary target is a primary probe that binds a primary target.

16. The method of claim 14, wherein the primary probe is a primary antibody or a nanobody.

17. The method of claim 15, wherein the primary probe is a primary antibody or a nanobody.

18. The method of claim 4, wherein the anti-hapten antibody is a primary antibody.

19. The method of claim 4, wherein the anti-hapten antibody comprises a primary antibody that binds the hapten and the method further comprises a secondary antibody (labeled with one or more reporter entities) that binds the primary antibody.

20. The method of claim 3, wherein the one or more deposited reporters is an auxiliary CARD-substrate labeled with an auxiliary hapten that mediates localization of auxiliary reporters in the vicinity of the target, and wherein signal is measured from one or more auxiliary reporters.

21. The method of claim 9, wherein the one or more deposited reporters is an auxiliary CARD-substrate labeled with an auxiliary hapten that mediates localization of auxiliary reporters in the vicinity of the target, and wherein signal is measured from one or more auxiliary reporters.

22. The method of claim 20, wherein the auxiliary CARD-substrate is a tyramide.

23. The method of claim 21, wherein the auxiliary CARD-substrate is a tyramide.

24. The method of claim 3, wherein the one or more deposited reporters is a fluorophore-labeled tyramide.

25. The method of claim 9, wherein the one or more deposited reporters is a fluorophore-labeled tyramide.