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(54) Title: AUTO-FEEDBACK LOOP BIOSENSOR

(57) Abstract: The present invention is predicated on a signal amplification auto-feedback loop for the detection of a target analyte in a sample. In the present invention, the presence of a target in a sample directly or indirectly releases a nuclease from aptamer-mediated inhibition. The nuclease is able, through acting on an intermediary nucleotide sequence, to release additional nucleases from inhibition which themselves release further nucleases in the same fashion. Each of the nucleases is also capable of generating a detectable signal. Due to the nature of an auto-feedback loop-generated signal, a small amount of target in a sample is able to rapidly generate an easily detectable signal in a short period of time.



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AUTO-FEEDBACK LOOP BIOSENSOR

5 PRIORITY CLAIM

This application claims priority to Australian provisional patent application 2008903043, filed 16 June 2008, the contents of which are hereby incorporated by reference.

10

FIELD

The present invention relates to methods and agents for the detection of a target in a sample. The methods and agents of the present invention exploit an auto-feedback
15 loop to provide signal amplification for the detection of the target in a sample.

BACKGROUND

A range of molecular technologies for the detection of a target analyte in a sample have
20 been developed. Such methods have application in, for example, public health, the detection of pathogens in food or water, epidemiological studies, genetically modified organism (GMO) detection, medicine, clinical diagnoses, disease susceptibility diagnoses, tissue typing, blood screening, forensic medicine, bioweapon detection, molecular toxicology, gene therapy, and DNA tagging, among many other
25 applications.

Current methods for detecting an analyte such as a nucleic acid generally involve one, or a combination of, molecular techniques. These techniques generally fall into three groups loosely defined as sequence-specific detection, sequence-specific enrichment
30 and signal amplification.

Most detection techniques gain their sequence specificity through base pairing of complementary probes or oligonucleotides to a sequence of interest within the target DNA sample.

5

The two most commonly used DNA detection methods, polymerase chain reaction (PCR) and Southern blotting, differ in how they proceed from this point. The PCR method enriches a target DNA through a series of amplification cycles and signal detection can be, for example, through the use of stains, fluorescence or radiolabeling.

10 Southern blotting involves no DNA enrichment step, but uses high-energy ^{32}P for signal amplification. These extensively used techniques, though highly developed, still retain significant drawbacks. For PCR, the equipment required is expensive, the process is time-consuming and the degree of expertise required is high. Southern blotting often uses hazardous radioactive labeling, takes up to a week to complete, and
15 requires large amounts of substrate DNA.

A biosensor for detecting analytes such as (but not limited to) nucleic acids which is target-specific and sensitive would be desirable. Furthermore, such a technique would also ideally require a low capital input (particularly in the case of equipment
20 requirement), minimal expertise or technique-specific training, and provide quick and accurate results.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the
25 common general knowledge in any country.

SUMMARY

The present invention is predicated on a signal amplification auto-feedback loop for
30 the detection of a target analyte in a sample. In the present invention, the presence of a

target in a sample directly or indirectly releases a nuclease from aptamer-mediated inhibition. The nuclease is able, through acting on an intermediary nucleotide sequence, to release additional nucleases from inhibition which themselves release further nucleases in the same fashion. Each of the nucleases is also capable of
5 generating a detectable signal. Due to the nature of an auto-feedback loop-generated signal, a small amount of target in a sample is able to rapidly generate an easily detectable signal in a short period of time.

Thus, in a first aspect, the present invention provides a method for detecting a target in
10 a sample, the method comprising:

providing a population of nuclease-aptamer complexes, each complex comprising a nuclease bound to an aptamer, wherein binding of the aptamer to the nuclease inhibits the activity of the nuclease;

providing an intermediary nucleotide sequence comprising: (i) a nucleotide
15 sequence which is digestible by a nuclease, and (ii) a trigger sequence which can hybridise with an aptamer in a nuclease-aptamer complex when the intermediary nucleotide sequence is digested by a nuclease, wherein hybridisation between the trigger sequence and an aptamer in a nuclease-aptamer complex reduces inhibition of the nuclease by the aptamer; and

20 applying the population of nuclease-aptamer complexes and the intermediary nucleotide sequence to the sample under conditions such that the presence of the target in the sample directly or indirectly reduces inhibition of a nuclease by an aptamer in a first nuclease-aptamer complex in the population;

wherein a nuclease having reduced inhibition by an aptamer digests an
25 intermediary nucleotide sequence such that hybridisation occurs between a trigger sequence and an aptamer in a second nuclease aptamer complex in the population to reduce inhibition of the second nuclease;

detecting nuclease activity in the sample, wherein increased nuclease activity is indicative of the presence of the target in the sample.

As set out above, the present invention is predicated, in part, on the use of an intermediary nucleotide sequence. When a target is present in a sample, inhibition of a nuclease by an aptamer in a first nuclease-aptamer complex in the population is reduced. This nuclease with reduced inhibition is then free to digest an intermediary
5 nucleotide sequence. As set out above, digestion of an intermediary nucleotide sequence releases the trigger sequence from the intermediary nucleotide sequence. The trigger sequence thus released can then hybridise with an aptamer in a second nuclease-aptamer complex in order to reduce inhibition of the nuclease therein. This second nuclease can then digest a further intermediary sequence, which in turn
10 releases a further trigger sequence, which in turn releases a further nuclease, and so forth. Thus, the intermediary nucleotide sequence allows the establishment of an auto-feedback loop wherein a single nuclease having reduced inhibition due to the presence of the target in a sample is able to activate many additional nucleases *via* the action of intermediary nucleotide sequences.

15

In some embodiments, the presence of the target in the sample reduces inhibition of a nuclease in a first nuclease-aptamer complex via a linker nucleotide sequence.

Thus, in some embodiments, the method of the present invention further comprises:

20 providing a linker nucleotide sequence comprising: (i) a binder sequence which can associate with a target if it is present in the sample, and (ii) a trigger sequence which can hybridise with an aptamer in a nuclease-aptamer complex when the binder sequence is associated with the target, wherein hybridisation between the trigger sequence and the aptamer reduces inhibition of the nuclease in the nuclease-aptamer
25 complex by the aptamer; and

applying the linker nucleotide sequence to the sample;

wherein the presence of the target in the sample reduces inhibition of a nuclease via association of the binder sequence with the target which allows hybridisation between the trigger nucleotide sequence and an aptamer in a first
30 nuclease aptamer complex to thus modify, reduce or eliminate binding between the

aptamer and the nuclease in the first nuclease-aptamer complex.

Notwithstanding the above, in some embodiments a linker nucleotide sequence is not used. Thus, in some embodiments, the presence of the target in the sample directly or indirectly reduces inhibition of a nuclease in a first nuclease-aptamer complex via hybridisation of the aptamer in the first nuclease-aptamer complex to all or part of the target to thus modify, reduce or eliminate binding between the aptamer and a nuclease in the first nuclease-aptamer complex.

- 10 In a second aspect, the present invention provides an isolated nucleic acid comprising an intermediary nucleotide sequence, the intermediary nucleotide sequence comprising: (i) a nucleotide sequence which is cleavable by a nuclease, and (ii) a trigger sequence which can hybridise with an aptamer in a nuclease-aptamer complex to reduce inhibition of the nuclease in the nuclease-aptamer complex by the aptamer when the intermediary nucleotide sequence is digested by a nuclease.

In some embodiments, the nucleic acid further comprises a binder sequence which can associate with a target.

- 20 In some embodiments, complete or partial digestion of the isolated nucleic acid by a nuclease is detectable. Thus, in some embodiments, the isolated nucleic acid molecule may also comprise a signalling nucleotide sequence.

- 25 In a third aspect, the present invention also provides an isolated nucleic acid molecule of the second aspect of the invention when used according to the first aspect of the invention.

- In a fourth aspect, the present invention also provides a kit for performing the method of the first aspect of the invention, the kit comprising an isolated nucleic acid according to the second aspect of the invention.
- 30

In a fifth aspect, the present invention also provides an RNase H-binding aptamer wherein binding of the aptamer to the RNase H inhibits the activity of the RNase H.

- 5 In a sixth aspect, the present invention provides an RNase H-aptamer complex comprising RNase H bound to an aptamer, wherein binding of the aptamer to the RNase H inhibits the activity of the RNase H in the RNase H-aptamer complex.

The present invention also provides a kit for performing the method of the first aspect
10 of the invention, the kit comprising an RNase H-aptamer complex.

Nucleotide and amino acid sequences are referred to herein by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers in the sequence listing, eg. < 400 > 1 (SEQ ID NO :1), < 400 > 2 (SEQ ID NO :
15 2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided at the end of the specification.

TABLE 1 – Summary of Sequence Identifiers

Sequence Identifier	Sequence
SEQ ID NO: 1	Aptamer 3_ADJ_L nucleotide sequence
SEQ ID NO: 2	Aptamer 3_ADJ_L trigger nucleotide sequence
SEQ ID NO: 3	<i>Eco</i> RI Intermediary nucleotide sequence
SEQ ID NO: 4	Tuberculosis linker nucleotide sequence
SEQ ID NO: 5	Synthetic tuberculosis target nucleotide sequence
SEQ ID NO: 6	Linker/intermediary nucleotide sequence
SEQ ID NO: 7	Linker nucleotide sequence with modified <i>Eco</i> RI recognition site
SEQ ID NO: 8	Target for linker/intermediary nucleotide sequence
SEQ ID NO: 9	Molecular break-light signalling molecule nucleotide sequence
SEQ ID NO: 10	GGGTGGGT aptamer nucleotide sequence motif

SEQ ID NO: 11	GGGGGGCATCG aptamer nucleotide sequence motif
SEQ ID NO: 12	Aptamer 3.8.4.1 nucleotide sequence
SEQ ID NO: 13	Aptamer 3.8 nucleotide sequence
SEQ ID NO: 14	Aptamer 3.9 nucleotide sequence
SEQ ID NO: 15	Aptamer 3.22 nucleotide sequence
SEQ ID NO: 16	Aptamer 5.10 nucleotide sequence
SEQ ID NO: 17	Aptamer 5.2 nucleotide sequence
SEQ ID NO: 18	Aptamer 5.3 nucleotide sequence
SEQ ID NO: 19	Aptamer 6.5 nucleotide sequence
SEQ ID NO: 20	Aptamer 6.6 nucleotide sequence
SEQ ID NO: 21	Aptamer 6.13 nucleotide sequence
SEQ ID NO: 22	Aptamer 3.8.4.1 trigger nucleotide sequence
SEQ ID NO: 23	Dengue type 3 linker nucleotide sequence
SEQ ID NO: 24	Synthetic dengue type 3 target nucleotide sequence
SEQ ID NO: 25	Dengue type 4 linker 1 nucleotide sequence
SEQ ID NO: 26	Dengue type 4 target 1 nucleotide sequence
SEQ ID NO: 27	Neomycin phosphotransferase linker nucleotide sequence
SEQ ID NO: 28	Synthetic neomycin phosphotransferase target nucleotide sequence
SEQ ID NO: 29	<i>Cre</i> recombinase linker nucleotide sequence
SEQ ID NO: 30	Synthetic <i>Cre</i> recombinase target nucleotide sequence
SEQ ID NO: 31	RNase H intermediary nucleotide sequence
SEQ ID NO: 32	Molecular break-light signalling molecule 1 nucleotide sequence
SEQ ID NO: 33	Molecular break-light signalling molecule 2 nucleotide sequence
SEQ ID NO: 34	Dengue type 4 linker 2 nucleotide sequence
SEQ ID NO: 35	Dengue type 4 target 2 nucleotide sequence
SEQ ID NO: 36	Multifunctional intermediary/signalling molecule nucleotide sequence

DESCRIPTION OF EXEMPLARY EMBODIMENTS

It is to be understood that the following description is for the purpose of describing particular embodiments only and is not intended to be limiting with respect to the
5 above description.

In a first aspect, the present invention provides a method for detecting a target in a sample, the method comprising:

providing a population of nuclease-aptamer complexes, each complex
10 comprising a nuclease bound to an aptamer, wherein binding of the aptamer to the nuclease inhibits the activity of the nuclease;

providing an intermediary nucleotide sequence comprising: (i) a nucleotide sequence which is cleavable by a nuclease, and (ii) a trigger sequence which can hybridise with an aptamer in a nuclease-aptamer complex when the intermediary
15 nucleotide sequence is digested by a nuclease, wherein hybridisation between the trigger sequence and an aptamer in a nuclease-aptamer complex reduces inhibition of the nuclease by the aptamer; and

applying the population of nuclease-aptamer complexes and the intermediary nucleotide sequence to the sample under conditions such that the presence of the target
20 in the sample directly or indirectly reduces inhibition of a nuclease by an aptamer in a first nuclease-aptamer complex in the population;

wherein a nuclease having reduced inhibition by an aptamer digests an intermediary nucleotide sequence such that hybridisation occurs between a trigger sequence and an aptamer in a second nuclease aptamer complex in the population to
25 reduce inhibition of the second nuclease;

detecting nuclease activity in the sample, wherein increased nuclease activity is indicative of the presence of the target in the sample.

The "sample" in which a target may be detected may be any sample that putatively
30 contains the target. For example, the sample may be a biological sample including

samples derived from an organism, a sample containing one or more cells, a blood sample, a plasma sample, a CSF fluid sample, an amniotic fluid sample and the like; an environmental sample such as a water, air or soil sample; a food or beverage sample; and the like. The samples contemplated herein may be used in a crude form, or the
5 samples may be processed for use in accordance with the present invention. For example, the sample may have one or more extraction or purification steps performed thereon in order to purify or semi-purify the target present in the sample.

As set out above, the method of the present invention is predicated, in part, on the use
10 of a “nuclease-aptamer complex”.

As referred to herein, a “nuclease” should be understood as any enzyme that can cleave the sugar-phosphate backbone of a nucleic acid. As such, the term “nuclease” should be understood to encompass both endonucleases and exonucleases.
15 Furthermore, the nucleases contemplated for use in accordance with the present invention may be deoxyribonucleases (which cleave DNA) or ribonucleases (which cleave RNA). In some embodiments, the nuclease used in accordance with the present invention is a nuclease that, once inhibition by the aptamer has been reduced or eliminated, still cannot cleave or digest the target, but can cleave or digest another
20 nucleotide sequence (such as a reporter nucleotide sequence – see later) such that the activity of the nuclease may be detected. Nucleases that may be used in accordance with the present invention include, for example, restriction endonucleases, nucleases that cleave at sequence mis-matches, S1 nuclease, T7 endonuclease I, T4 endonuclease VII, CEL I (a plant-specific extracellular glycoprotein that belongs to the S1 nuclease
25 family), and ribonucleases such as RNase A or RNase H.

In some embodiments (as described hereafter) the nuclease may be a restriction endonuclease such as *EcoRI*.

30 In some embodiments (as described hereafter), the nuclease may be an RNase such as

RNase H.

An active nuclease is able to specifically cleave its desired substrate. In the case of an RNase such as RNase H, that substrate may be an RNA portion of a DNA/RNA hybrid (i.e. an RNA sequence in complement with a DNA sequence). Similarly, the substrate for a particular restriction endonuclease may be the enzyme's specific recognition sequence within (generally) double-stranded DNA. The intermediary oligonucleotides and the signalling molecules for use in the present invention (described later) may comprise substrates of the respective nucleases.

The term "aptamer", as referred to herein, should be understood as a nucleic acid molecule, at least a portion of which is able to bind to another molecule. Nucleic acid aptamers are generally single-stranded nucleic acid molecules with complex secondary or tertiary structures (which may include double-stranded portions or regions) that can specifically bind a target molecule with high affinity. When bound to enzymes, certain aptamers are able to reduce or inhibit their enzymatic activity. Generally, the aptamers contemplated by the present invention can bind at least to a nuclease, and thus alter the activity of the nuclease. Furthermore, the nuclease-binding aptamers contemplated by the present invention generally reduce or eliminate the activity of the nuclease when bound thereto. The aptamers of the present invention may also comprise a region which does not bind to the nuclease. This region, or the nuclease binding region itself, may also exhibit binding affinity toward another molecule such as a target or linker nucleotide sequence (as described later).

The aptamers contemplated for use in accordance with the present invention may be any suitable nucleic acid or equivalent thereof. In this regard, the aptamers may comprise, for example, DNA, RNA, a nucleic acid mimic such as Peptide Nucleic Acid (PNA) or Locked Nucleic Acid (LNA), DNA or RNA comprising one or more modified nucleotides, and the like. "Modified" nucleotides include, for example, nucleotides having chemical modifications to any of the phosphate backbone, sugar moiety or base

moiety of the nucleotide, tritylated bases and unusual bases such as inosine. The use of modified nucleotides may also affect the binding characteristics of the aptamer to the nuclease, for example as described in Latham *et al.* (*Nucl Acids Res* 22(14): 2817-2822, 1994).

5

In some embodiments RNA aptamers may be used, since RNA can form secondary structures that DNA generally does not, such as pseudoknots and base triples.

Nucleic acid aptamers may also be modified, for example to increase stability, in a number of ways including, for example:

- (i) Synthesis of aptamers using L-nucleotides (the mirror image of natural nucleotides) so that they cannot be degraded by naturally occurring nucleases;
- 15 (ii) Incorporation of locked nucleic acid (LNA) and/or peptide nucleic acid (PNA) residues into the aptamer. LNAs and PNAs also increase stability of nucleic acid duplexes;
- (iii) Other chemical modifications of ribonucleotides, such as 2'-amino- and 2'-fluoro-pyrimidine nucleotides or 2'-O-methyl nucleotides; and/or
- 20 (iv) Capping at the 3' end with a deoxythymidine to increase resistance to exonuclease degradation.

Nucleic acid aptamers that bind to, and inhibit the activity of, a particular protein (such as a nuclease) may be produced using methods known in the art. For example, *in-vitro* selection methods (eg. see Ellington and Szostak, *Nature* 346(6287): 818-22, 1990) and SELEX methods (eg. see Tuerk and Gold, *Science* 249(4968): 505-510, 1990) may be used. Further details relating to the production and selection of aptamers may also be found in the review of Osborne and Ellington (*Chem Rev* 97(2): 349-370, 1997).

30 In light of the above, a "nuclease-aptamer complex" should be understood as a

nuclease to which an aptamer is bound, or a nuclease and aptamer which may become bound under the conditions under which the method is performed, such that binding of the aptamer to the nuclease inhibits the activity of the nuclease and inhibition of the nuclease's activity by the aptamer is directly or indirectly reduced or eliminated when
5 the target is present in the sample.

As set out above, the present invention is also predicated, in part, on the use of an intermediary nucleotide sequence. The intermediary nucleotide sequence comprises: (i) a nucleotide sequence which is digestible by a nuclease, and (ii) a trigger sequence
10 which can hybridise with an aptamer in a nuclease-aptamer complex when the intermediary nucleotide sequence is digested by a nuclease, wherein hybridisation between the trigger sequence and an aptamer in a nuclease-aptamer complex reduces inhibition of the nuclease by the aptamer.

15 When a target is present in a sample, inhibition of a nuclease by an aptamer in a first nuclease-aptamer complex in the population is reduced. This nuclease with reduced inhibition is then free to digest an intermediary nucleotide sequence. As set out above, digestion of an intermediary nucleotide sequence releases the trigger sequence from the intermediary nucleotide sequence. The trigger sequence thus released can then
20 hybridise with an aptamer in a second nuclease-aptamer complex in order to reduce inhibition of the nuclease therein. This second nuclease can then digest a further intermediary sequence, which in turn releases a further trigger sequence, which in turn releases a further nuclease, and so forth. Thus, the intermediary nucleotide sequence allows the establishment of an auto-feedback loop wherein a single nuclease having
25 reduced inhibition due to the presence of the target in a sample is able to activate many additional nucleases via the action of the intermediary nucleotide sequence.

Reference herein to digestion of a nucleotide sequence should be understood to include partial and/or complete digestion of the nucleotide sequence. Digestion of a nucleotide
30 sequence need not be complete digestion of a nucleotide sequence to individual

nucleotides.

As set out above, the presence of the target in the sample directly or indirectly reduces inhibition of a nuclease by an aptamer in a first nuclease-aptamer complex in the population. In some embodiments, the presence of the target in the sample directly or indirectly modifies, reduces or eliminates binding between the nuclease and the aptamer, thereby reducing or eliminating inhibition of the nuclease's activity by the aptamer.

As referred to herein modification, reduction or elimination of binding between the nuclease and the aptamer refers to any qualitative or quantitative change in the nature of the binding between the aptamer and the nuclease which brings about a reduction or elimination of inhibition of the nuclease's activity by the aptamer. Such changes may arise as a result of binding between the aptamer and another molecule such as the target or a linker nucleotide sequence or binding between the nuclease-aptamer complex and another molecule such as the target or a linker nucleotide sequence.

"Qualitative or quantitative changes" in the nature of the binding between the aptamer and the nuclease may include, for example: a change in the binding strength between the nuclease and the aptamer; a change in the interaction or binding sites on the nuclease and/or aptamer; dissociation of the nuclease-aptamer complex, optionally with binding of the aptamer to another molecule; a change in the conformation, secondary structure or tertiary structure of the aptamer or nuclease which reduces the level of inhibition of the nuclease by the aptamer; a change in the relative positions of the binding sites on the aptamer and/or nuclease; and the like.

In some embodiments, the presence of the target in the sample reduces inhibition of a nuclease in a first nuclease-aptamer complex via a linker nucleotide sequence.

Thus, in some embodiments, the method of the present invention further comprises:

providing a linker nucleotide sequence comprising: (i) a binder sequence which can associate with a target if it is present in the sample, and (ii) a trigger sequence which can hybridise with an aptamer in a nuclease-aptamer complex when the binder sequence is associated with the target, wherein hybridisation between the trigger sequence and the aptamer reduces inhibition of the nuclease in the nuclease-aptamer complex by the aptamer; and

applying the linker nucleotide sequence to the sample;

wherein the presence of the target in the sample reduces inhibition of a nuclease via association of the binder sequence with the target which allows hybridisation between the trigger nucleotide sequence and an aptamer in a first nuclease aptamer complex to thus modify, reduce or eliminate binding between the aptamer and the nuclease in the first nuclease-aptamer complex.

In the above embodiments, nuclease inhibition is effected by the bound aptamer in the nuclease-aptamer complex and the trigger sequence is designed to hybridise to the aptamer to modify, reduce or eliminate binding between the aptamer and the nuclease when the binder sequence is associated with the target.

In some embodiments, the linker nucleotide sequence is comprised within a nucleic acid having a stem-loop secondary structure and all or part of the binder sequence is comprised within a loop of the stem-loop structure and all or part of the trigger sequence is comprised within a stem of the stem-loop structure.

A "stem-loop secondary structure" as referred to herein may comprise one or more double stranded stem portions and one or more regions of unpaired nucleotide residues forming a loop portion.

In some embodiments, the trigger portion is generally of a length sufficient to enable denaturation or dissociation of a stem portion when the linker binds to the target. That is, hybridisation of the binder generally effects denaturation or dissociation of a stem

portion of the linker, thus exposing the aptamer-binding region(s) of the linker nucleotide sequence. In light of the above, a stem-portion of the stem loop structure generally comprises at least 5 nucleotides and may extend to about 50 nucleotides in length. However, the present invention should not be considered limited to any
5 specific length of sequence and other sequence lengths may be used that provide the functionality described above.

In some embodiments, the linker is designed such that the melting temperature of the binder, when hybridised to the target, is higher than the melting temperature of the
10 stem of the linker. In some embodiments, the melting temperature of the binder sequence when hybridised to the target is about 1°C, about 2°C, about 3°C, about 4°C, about 5°C, about 6°C, about 7°C, about 8°C, about 9°C, about 10°C, about 11°C, about 12°C, about 13°C, about 14°C, about 15°C, about 16°C, about 17°C, about 18°C, about 19°C or about 20°C higher than the melting temperature of the stem of the linker. In
15 some embodiments, the melting temperature of the binder sequence when hybridised to the target is about 7°C higher than the melting temperature of the stem of the linker.

In some embodiments when the target is present in the sample, a heat or chemically denatured linker binds to the target (via the binder sequence) in preference to reverting
20 to its native stem/loop structure following heat or chemical denaturation followed by the introduction of renaturation conditions.

The stem-portion of the stem-loop structure may be completely homologous, ie. no mismatches in the hybridising sequences. Alternatively, the stem portion may include
25 one or more mis-matched base pairs. For example, mis-matches may be useful in promoting denaturation of the stem portion of the stem loop structure during the method of the present invention and/or to allow hybridisation between the aptamer and the trigger sequence in the stem portion. Features such as mismatches and/or overhangs may also be incorporated into the stem to provide an appropriate melting
30 temperature difference to be obtained without the requirement for an excessively long

target-complementary sequence.

In some embodiments, the binder sequence may not be wholly within a loop, and may also be part of the stem. Similarly, the trigger sequence may not necessarily be wholly
5 contained within a stem portion, and may extend into a loop portion

In some embodiments, the binder and trigger portions of the linker may be separated by one or more nucleotide residues, partially overlapping, completely overlapping or one region may be contained within the other.

10

The use of a linker nucleotide sequence in accordance with this embodiment also provides a significant advantage in that it removes the requirement to design or select an aptamer to have a target binding region. Rather, the target binding region can be incorporated into the binder sequence of the linker and the trigger sequence of the
15 linker may be designed to bind to the aptamer. This provides an advantage in that incorporation of an aptamer-binding trigger sequence into a linker is simpler and/or less time-consuming than having to design or select an aptamer to include both a nuclease-binding region and a target binding region. That is, the trigger sequence may be designed such that it binds to an aptamer that binds to a nuclease rather than
20 having to select an aptamer on the basis of the ability to bind to both a nuclease and a target. Another significant advantage is that a single nuclease-aptamer complex may be applied to the detection of many different targets, simply by modifying the binder sequence in the linker.

25 In some embodiments, the intermediary nucleotide sequence and the linker nucleotide sequence are comprised within one contiguous nucleic acid molecule. In some embodiments, the intermediary nucleotide sequence is comprised within a stem of the stem-loop structure.

30 Linker nucleotide sequences which are able to interact with a range of targets may be

readily generated using methods known in the art. Linker nucleotide sequences that may be used in accordance with the present invention include, for example, nucleic acids comprising a nucleotide sequence selected from the list consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29 and
5 SEQ ID NO: 34.

Notwithstanding the above, in some embodiments a linker nucleotide sequence is not used. Thus, in some embodiments, the method of the present invention may exploit hybridisation between an aptamer in a nuclease-aptamer complex and the target in
10 order to modify, reduce or eliminate binding between the nuclease and the aptamer.

In these embodiments, the aptamer is able to hybridise to the target to modify, reduce or eliminate binding between the nuclease and the aptamer. Hybridisation between the target and the aptamer, together with concomitant modification, reduction or
15 elimination of binding between the nuclease and aptamer, may occur under the initial conditions of the sample or, alternatively, the conditions may be modified to allow or promote binding between the aptamer and the target (if it is present in the sample).

For example, binding between the aptamer and the target and/or binding between the
20 aptamer and nuclease may be modulated by altering the salt concentration and/or temperature. In addition, destabilising agents such as formamide may also be used to modulate the binding of the aptamer to any of the nuclease, the target and/or a linker nucleotide sequence.

25 In this embodiment, the aptamer should be designed or selected such that it comprises both a region which binds to the nuclease and a region which can hybridise, at least under some hybridisation conditions, to the target. These regions may be separated by one or more nucleotide residues, partially overlapping, completely overlapping or one region may be contained within the other.

Accordingly, in some embodiments, the presence of the target in the sample directly or indirectly reduces inhibition of a nuclease in a first nuclease-aptamer complex via hybridisation of the aptamer in the first nuclease-aptamer complex to all or part of the target to thus modify, reduce or eliminate binding between the aptamer and a nuclease
5 in the first nuclease-aptamer complex.

As set out above, in some embodiments, the nuclease may be a restriction endonuclease.

10 As set out above, in some embodiments, the nuclease used in accordance with the present invention is a restriction endonuclease. As referred to herein, a “restriction endonuclease” refers to any endonuclease that binds to double-stranded DNA at a specific nucleotide sequence and then, if both strands of the DNA lack appropriate modification at that sequence, cleaves the DNA either at the recognition sequence or at
15 another site in the DNA molecule. A wide array of restriction endonucleases with different recognition sites and different cleavage sites would be readily ascertained by one of skill in the art. For example, a range of restriction endonucleases may be sourced from New England Biolabs (Ipswich, MA, USA).

20 In some embodiments, the restriction endonuclease may be *EcoRI*.

In some embodiments wherein the nuclease is *EcoRI*, the aptamer in the nuclease-aptamer complex may comprise the nucleotide sequence set forth in SEQ ID NO: 1 or a variant of said aptamer which retains the ability to bind to and inhibit the activity of
25 *EcoRI*.

In some embodiments wherein the nuclease is *EcoRI*, the trigger sequence in the intermediary and/or linker nucleotide sequences comprises the nucleotide sequence set forth in SEQ ID NO: 2.

In some embodiments wherein the nuclease is *EcoRI*, the intermediary nucleotide sequence comprises the nucleotide sequence set forth in SEQ ID NO: 3.

Further examples of suitable restriction endonucleases binding aptamers, including
5 *EcoRI* binding aptamers, are also described in WO 2008/122088, the contents of which is hereby incorporated by reference.

In some embodiments, the nuclease used in accordance with the present invention is an RNase. In some embodiments the RNase is an RNase H.

10

RNase H (EC 3.1.26.4) is a ribonuclease that cleaves the 3'-O-P-bond of RNA in a DNA/RNA duplex to produce 3'-hydroxyl and 5'-phosphate terminated products. RNase H is a non-specific endonuclease and catalyzes the cleavage of RNA via a hydrolytic mechanism, aided by an enzyme-bound divalent metal ion.

15

In some embodiments wherein the nuclease is RNase H, the aptamer in the nuclease-aptamer complex may comprise the nucleotide sequence motif set forth in SEQ ID NO: 10.

20 In some embodiments, the aptamer comprises the nucleotide sequence set forth in SEQ ID NO: 12; or a variant of said aptamer which retains the ability to bind to and inhibit the activity of RNase H.

In some embodiments, the aptamer comprises a nucleotide sequence selected from the
25 group consisting of SEQ ID NO: 13, SEQ ID NO: 14 and/or SEQ ID NO: 15; or a variant of said aptamer which retains the ability to bind to and inhibit the activity of RNase H.

In some embodiments wherein the nuclease is RNase H, the aptamer in the nuclease-aptamer complex may comprise the nucleotide sequence motif set forth in SEQ ID NO:
30 11.

In some embodiments, the aptamer comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20 and/or SEQ ID NO: 21; or a variant of said aptamer which retains the
5 ability to bind to and inhibit the activity of RNase H.

In some embodiments wherein the nuclease is RNase H, the trigger sequence in the intermediary and/or linker nucleotide sequences comprises the nucleotide sequence set forth in SEQ ID NO: 22.
10

In some embodiments wherein the nuclease is RNase H the intermediary nucleotide sequence comprises the nucleotide sequence set forth in SEQ ID NO: 31.

As set out above, the methods of the present invention involve detecting the activity of
15 the nuclease, wherein increased nuclease activity is indicative of the presence of the target in the sample.

The method used for detecting the activity of the nuclease may be any suitable method for the subject nuclease. In some embodiments, the activity of the nuclease is
20 determined by the rate or extent of digestion of a reporter nucleotide sequence.

For example, the activity of a DNase or RNase may be ascertained by observing degradation, cleavage or digestion of a reporter nucleotide sequence comprising DNA or an analog thereof, RNA or an analog thereof or both DNA or an analog thereof and
25 RNA or an analog thereof.

The reporter nucleotide sequence may be single-stranded or double-stranded, as appropriate for the activity of the nuclease. Cleavage of the reporter nucleotide sequence may be detected by any known method. For example, cleavage of a reporter
30 nucleotide sequence into a lower molecular weight product may be determined by

electrophoretic methods, staining methods, the release of a labelled nucleotide, cleavage of a fluorophore/quencher labelled nucleic acid to release a fluorophore, and the like.

- 5 The reporter nucleotide sequence may be comprised within a separate reporter nucleic acid. Alternatively, the reporter nucleotide sequence may be comprised within the same nucleic acid molecule as the intermediary nucleotide sequence and/or the linker nucleotide sequence.
- 10 In some embodiments, a fluorophore is bound to the reporter nucleotide sequence and a quencher, which quenches the fluorescence of the fluorophore, is also bound to the reporter nucleotide sequence, wherein digestion of all or part of the reporter nucleotide sequence reduces or eliminates the quenching of the fluorophore by the quencher.
- 15 Exemplary fluorophores and quenchers would be readily ascertained by one of skill in the art. In this regard, reference is made to the review of Marras (*Methods Mol Biol* 335, 3-16, 2006).

In embodiments utilising a restriction endonuclease, the activity of the restriction
20 endonuclease may be determined by the rate or extent of cleavage of a reporter nucleotide sequence which comprises at least a region of double stranded DNA.

In the embodiments utilising an RNase H, the activity of the RNase H may be determined by the rate or extent of cleavage of a reporter nucleotide sequence which
25 comprises a DNA/RNA duplex.

In some embodiments, wherein the reporter nucleotide sequence comprises both DNA or an analog thereof and RNA or an analog thereof, the fluorophore may be bound to an RNA portion of the reporter nucleotide sequence and the quencher may be bound
30 to a DNA portion of the reporter nucleotide sequence or, alternatively, the fluorophore

may be bound to a DNA portion of the reporter nucleotide sequence and the quencher may be bound to an RNA portion of the reporter nucleotide sequence.

5 In the above embodiments, digestion of part of the reporter nucleotide sequence allows dissociation of the fluorophore and quencher and thus allows the generation of a signal by the fluorophore.

10 In some embodiments, the reporter nucleotide sequence is comprised within a Molecular Break-Light nucleic acid molecule as described by Biggins *et al.* (*Proc Natl Acad Sci USA* 97(25): 13537–13542, 2000)

15 In some embodiments, a polypeptide is bound to the reporter nucleotide sequence and an immobilisable agent is bound to the reporter nucleotide sequence, wherein cleavage of the reporter nucleotide sequence releases the polypeptide from the immobilisable agent; such that after cleavage of the reporter nucleotide sequence and immobilisation of the immobilisable agent, the amount of non-immobilised polypeptide is indicative of the activity of the nuclease.

20 A wide array of “immobilisable agents” would be readily ascertained by one of skill in the art and may include, for example:

- (i) an antigen, which may be immobilised by contacting with an immobilised antibody that can bind the antigen;
- (ii) an antibody, which may be immobilised by contacting with an immobilised antigen or anti-idiotypic antibody that can bind the antibody;
- 25 (iii) a polypeptide comprising a histidine tag, which may be immobilised by contacting an affinity medium comprising nickel or cobalt ions;
- (iv) biotin, which may be immobilised by contacting with immobilised avidin or streptavidin;
- 30 (v) avidin or streptavidin, which may be immobilised by contacting with

immobilised biotin; and/or

- (vi) a magnetic or paramagnetic particle, which may be immobilised via a magnetic field.

5 As set out above, some immobilisable agents may be immobilised by contacting the immobilisable agent with a binding partner that is itself immobilised. The immobilisation of the binding partner may be achieved using any means known in the art. For example, the binding partner of the immobilisable agent may be immobilised onto a surface of a culture vessel, tube or plate (which may have been pre-treated with
10 an agent such as a silane), immobilised onto the surface of a bead or other particle, immobilised onto a column or other chromatography medium, immobilised onto a membrane, or immobilised onto a solid substrate suitable for an array.

A range of other immobilisable agents would also be readily ascertained by one of skill
15 in the art, and the present invention should not be considered in any way limited to the immobilisable agents exemplified above.

In some embodiments, the immobilisable agent is a magnetic bead and immobilisation of the immobilisable agent is effected by the application of a magnetic field to the
20 sample.

After immobilisation of the immobilisable agent, any polypeptide remaining “free” in the sample may be detected using any standard methods of protein detection, as are known in the art such as electrophoresis, immunochromatographic tests, including
25 lateral flow strips, western blotting, mass spectroscopy, detection using a biosensor (for a review of biosensor-based detection of proteins in solution see Leca-Bouvier and Blum, *Analytical Letters* 38(10): 1491-1517, 2005). A range of exemplary protein detection methods may be found in *Proteins and Proteomics: A Laboratory Manual* (Simpson, CSHL Press, 2003).

In the case of polypeptides with detectable activity, such as enzymatic activity, the polypeptide may be detected by detection of the activity of the polypeptide. Suitable polypeptides with detectable enzymatic activity may include, for example, peroxidases such as HRP, glucorinidases such as GUS and galactosidases such as beta-galactosidases.

As set out above, the present invention contemplates a method for detecting a “target” in a sample.

The target which is detectable by the methods of the present invention may be any target to which either a binder sequence or an aptamer in a nuclease-aptamer complex can associate with.

In some embodiments, the target may be a nucleic acid. In these embodiments, a binder sequence in a linker nucleotide sequence may hybridise with the target, or an aptamer in a nuclease-aptamer complex may hybridise with the target in order to reduce inhibition of a nuclease by an aptamer in a nuclease-aptamer complex.

In addition to specifically detecting a target nucleic acid, the present invention is also amenable to detecting other molecules.

For example an aptamer in a nuclease-aptamer complex may be a bifunctional aptamer which binds to a nuclease and also to a target. In these embodiments, binding of the aptamer to the target typically reduces inhibition of the nuclease by the aptamer. In some embodiments, the aptamer in a nuclease-aptamer complex is an allosteric aptamer that binds to a nuclease (to form a nuclease-aptamer complex), but which also associates with a target. When associated with the target, the allosteric aptamer may change conformation and release the nuclease in the nuclease-aptamer complex from inhibition. For example, Yoshida *et al.* (*Biotechnology Letters* 30: 421-425, 2008) describe a system that can detect the presence of immunoglobulin E (IgE) using a linked IgE-

binding/thrombin-inhibiting aptamer. A detectable signal is induced *via* a conformational change in the thrombin-inhibiting moiety through the binding of an IgE molecule to the IgE-binding moiety. Similarly, modification of an aptamer in a nuclease-aptamer complex of the present invention, whereby an analyte induces a conformational change in the aptamer, could also be used to release a nuclease from inhibition in a nuclease-aptamer complex.

In some embodiments, the binder sequence in a linker nucleotide sequence may bind to a non-nucleic acid target. In some embodiments, the binder sequence may comprise an aptamer which binds to a target. Again, in these embodiments, association of a binder sequence with the target allows hybridisation between the trigger nucleotide sequence and an aptamer in a first nuclease aptamer complex to thus modify, reduce or eliminate binding between the aptamer and the nuclease in the first nuclease-aptamer complex.

In light of the above, the target may be any target to which an aptamer and/or a binder nucleotide sequence can associate with. In this regard, the target may include, for example:

- (i) a nucleic acid;
- (ii) a polypeptide;
- (iii) a carbohydrate, for example, biotin, cellobiose or sephadex;
- (iv) an inorganic molecule, for example, such as vitamin B12, a metal ion or malachite green;
- (v) an organic molecule, for example, dopamine, tobramycin, L-tyrosinamide, flavin mononucleotide, cocaine, moenomycin A, theophylline, AMP, hematoporphyrin, an amino acid, cyanocobalamin or cholic acid;
- (vi) an aminoglycoside, for example, lectin, streptomycin, tetracycline, neomycin B; or
- (vii) other targets, for example, anthrax spores or rat brain tumour microvessels.

In a second aspect, the present invention provides an isolated nucleic acid comprising an intermediary nucleotide sequence, the intermediary nucleotide sequence comprising: (i) a nucleotide sequence which is digestible by a nuclease, and (ii) a trigger sequence which can hybridise with an aptamer in a nuclease-aptamer complex to reduce inhibition of the nuclease in the nuclease-aptamer complex by the aptamer when the intermediary nucleotide sequence is digested by a nuclease.

The intermediary sequence contemplated in this aspect of the invention may be as hereinbefore described with reference to the first aspect of the invention.

In some embodiments, the nucleic acid further comprises a binder sequence which can associate with a target.

In these embodiments, the binder sequence may be as hereinbefore described with reference to the first aspect of the invention. In this embodiment, the isolated nucleic acid molecule may provide the functions of the intermediary nucleotide sequence and the linker nucleotide sequence when used in a method according to the first aspect of the invention. In these embodiments, the trigger nucleotide sequence from the intermediary nucleotide sequence and the trigger sequence from the linker nucleotide sequence may be the same nucleotide sequence in the isolated nucleic acid.

In some embodiments, complete or partial digestion of the isolated nucleic acid by a nuclease is detectable. Thus, in this embodiment, the isolated nucleic acid molecule may also comprise a signalling nucleotide sequence as hereinbefore described.

In some embodiments, the isolated nucleic acid comprises a fluorophore and a quencher that quenches the fluorescence of the fluorophore and wherein complete or partial digestion of the nucleic acid reduces or eliminates quenching of the fluorophore by the quencher.

In this embodiment, the isolated nucleic acid may comprise both the intermediary sequence and the signalling nucleotide sequence. In some embodiments, the isolated nucleic acid may comprise each of the intermediary nucleotide sequence, the linker nucleotide sequence and the signalling nucleotide sequence.

5

The isolated nucleic acid of the second aspect of the invention may comprise DNA or an analog thereof, RNA or an analog thereof or comprise a hybrid of DNA or an analog thereof and RNA or an analog thereof. The composition of the isolated nucleic acid thus may be determined on the basis of a nuclease with which the isolated nucleic acid is to be used and/or the nature of any signalling nucleotide sequence incorporated into the isolated nucleic acid.

In some embodiments wherein the isolated nucleic acid comprises a signalling nucleotide sequence, a fluorophore may be bound to an RNA portion of the isolated nucleic acid and a quencher may be bound to a DNA portion of the isolated nucleic acid or, alternatively, a fluorophore may be bound to a DNA portion of the isolated nucleic acid and a quencher may be bound to an RNA portion of the isolated nucleic acid.

20 In some embodiments, the isolated nucleic acid comprises a trigger sequence comprising the nucleotide sequence set forth in SEQ ID NO: 2.

In some embodiments, the isolated nucleic acid comprises an intermediary nucleotide sequence comprising the nucleotide sequence set forth in SEQ ID NO: 3.

25

In some embodiments the isolated nucleic acid comprises a trigger sequence comprising the nucleotide sequence set forth in SEQ ID NO: 22.

In some embodiments the isolated nucleic acid comprises an intermediary nucleotide sequence comprising the nucleotide sequence set forth in SEQ ID NO: 31.

30

In a third aspect, the present invention also provides an isolated nucleic acid molecule of the second aspect of the invention when used according to the first aspect of the invention.

5

In a fourth aspect, the present invention also provides a kit for performing the method of the first aspect of the invention, the kit comprising an isolated nucleic acid according to the second aspect of the invention.

10 In addition to the isolated nucleic acid according to the second aspect of the invention, the kit may also comprise one more additional reagents needed to perform the method of the first aspect of the invention. Instructions for performing the method, and/or suitable reaction vessels may also be included in the kit.

15 In some embodiments, the kit according to the fourth aspect of the invention may further comprise a population of nuclease-aptamer complexes. The population of nuclease-aptamer complexes may include a nuclease-aptamer complex as hereinbefore described with reference to the first aspect of the invention.

20 In a fifth aspect, the present invention provides an RNase H-binding aptamer wherein binding of the aptamer to the RNase H inhibits the activity of the RNase H.

In some embodiments, the RNase H-binding aptamer comprises the nucleotide sequence motif set forth in SEQ ID NO: 10.

25

In some embodiments, the RNase H-binding aptamer comprises the nucleotide sequence set forth in SEQ ID NO: 12; or a variant of said aptamer which retains the ability to bind to and inhibit the activity of RNase H.

30 In some embodiments the RNase H-binding aptamer comprises a nucleotide sequence

selected from the group consisting of SEQ ID NO: 13, SEQ ID NO: 14 and/or SEQ ID NO: 15; or a variant of said aptamer which retains the ability to bind to and inhibit the activity of RNase H.

- 5 In some embodiments, the RNase H-binding aptamer comprises the nucleotide sequence motif set forth in SEQ ID NO: 11.

- In some embodiments, the RNase H-binding aptamer comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18,
10 SEQ ID NO: 19, SEQ ID NO: 20 and/or SEQ ID NO: 21; or a variant of said aptamer which retains the ability to bind to and inhibit the activity of RNase H.

- In a sixth aspect, the present invention also provides an RNase H-aptamer complex comprising RNase H bound to an aptamer, wherein binding of the aptamer to the
15 RNase H inhibits the activity of the RNase H in the RNase H-aptamer complex.

In some embodiments, the aptamer in the RNase H-aptamer complex is an aptamer according to the fifth aspect of the invention.

- 20 As will be appreciated, the RNase H-aptamer complex may be used in accordance with at least some embodiments of the method described herein. As such, the present invention also provides a method according to the first aspect of the invention wherein the nuclease-aptamer complex is an RNase H-aptamer complex according to the sixth aspect of the invention.

25

In a seventh aspect, the present invention also provides a kit for performing the method of the first aspect of the invention, the kit comprising an RNase H-aptamer complex according to the sixth aspect of the invention.

- 30 In addition to the isolated nucleic acid according to the second aspect of the invention,

the kit may also comprise one or more additional reagents needed to perform the method of the first aspect of the invention. Instructions for performing the method, and/or suitable reaction vessels may also be included in the kit.

- 5 In some embodiments, the kit may also comprise a nucleic acid molecule according to the second aspect of the invention, as hereinbefore described.

Finally, reference is made to standard textbooks of molecular biology that contain methods for carrying out basic techniques encompassed by the present invention,
10 including DNA restriction and ligation for the generation of the various genetic constructs described herein. See, for example, Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York, 1982) and Sambrook *et al.* (2000, *supra*).

- 15 The present invention is further described by the following non-limiting examples:

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of an embodiment of a restriction endonuclease-
20 based auto-feedback loop. The linker molecule switches from its inactive to its active state when hybridised to a target nucleic acid following denaturation and renaturation. The active linker binds to the restriction endonuclease-inhibiting aptamer and releases the restriction endonuclease from inhibition. The active restriction endonuclease then initiates an auto-feedback loop by cleaving an intermediary molecule which, in turn,
25 releases a further restriction endonuclease molecule from aptamer-mediated inhibition. Active restriction endonuclease molecules can specifically cleave multiple signalling molecules and generate a detectable signal.

Figure 2 shows embodiments of intermediary molecules. A. Shows an intermediary
30 molecule for the Apt₃_ADJ/*Eco*RI complex. The molecule contains the *Eco*RI

recognition site GAATTC (shaded blue) and is cleaved at G/AATTC in each strand (red line). The trigger moiety that binds to the *EcoRI*-inhibiting aptamer 3_ADJ_L is shaded red. The trigger moiety's complement contains two mismatches with the trigger which, along with an overhanging trigger, reduces its melting temperature to below reaction temperatures following *EcoRI* cleavage. B. Following cleavage by *EcoRI*, the trigger moiety melts away from its complement and is able to bind to aptamer 3_ADJ_L and release *EcoRI* from inhibition. C. Shows a combined linker and intermediary molecule. The molecule contains a target-complementary portion (shaded pink) in addition to the trigger moiety and restriction endonuclease recognition site contained in the discrete intermediary. Following denaturation, the molecule is able to specifically bind to a target nucleic acid and, subsequently, allow the exposed trigger moiety to release *EcoRI* from aptamer-mediated inhibition. Non-target bound renatured molecules can act as an intermediary as described above.

Figure 3 shows the results of an embodiment of a trigger-induced auto-feedback loop using a discrete intermediary molecule and an *EcoRI*/aptamer complex. The reaction was initiated by the addition of oligonucleotide trigger molecules which, in turn, released *EcoRI* molecules from inhibition. The presence of discrete intermediaries resulted in a significant increase in the rate at which a signal was generated compared to the control reaction where no intermediaries were added.

Figure 4 shows the results of an embodiment of a target nucleic acid-induced auto-feedback loop using a discrete intermediary molecule and an *EcoRI*/aptamer complex. The reaction was initiated by the binding of linkers to synthetic tuberculosis genome-derived oligonucleotides which, in turn, released *EcoRI* molecules from inhibition. The presence of discrete intermediaries resulted in a significant increase in the rate at which a signal was generated compared to the control reaction where no intermediaries were added. Due to a small portion of active *EcoRI* molecules present in the 'no target' control, the 'no target' signal was also amplified to a degree.

Figure 5 shows the results of an embodiment of a target nucleic acid-induced auto-feedback loop using a combined linker/intermediary molecule and an *EcoRI*/aptamer complex. The reaction was initiated by the binding of linker/intermediary molecules to synthetic target oligonucleotides. The presence of renatured, non-target bound, linker/intermediaries resulted in a significant increase in the rate at which a signal was generated compared to the control reaction where no molecules with an intermediary function were added. Due to a small portion of active *EcoRI* molecules present in the 'no target' control, the 'no target' signal was also amplified to a degree.

Figure 6 is a schematic representation of an embodiment of an RNase H-based system with a multifunctional linker/intermediary molecule and a separate signalling molecule. In its active state following specific hybridisation to a target nucleic acid, the linker/intermediary is able to release RNase H from aptamer-mediated inhibition. Active RNase H can cleave the RNA portion of the linker/intermediary and leave the single-stranded trigger moiety exposed. Subsequently, the trigger moiety can release an additional RNase H molecule from aptamer-mediated inhibition which, in turn, can release further molecules, thus forming an auto-feedback loop. Active RNase H can also cleave the RNA portion of a DNA/RNA hybrid signalling molecule and produce a detectable signal through the physical separation of the molecule's fluorophore and quencher.

Figure 7 is a schematic representation of an embodiment of an RNase H-based system with separate linker, intermediary and signalling molecules. In its active state following specific hybridisation to a target nucleic acid, the linker is able to release RNase H from aptamer-mediated inhibition. Active RNase H can cleave the RNA portion of the intermediary and leave the single-stranded trigger moiety exposed. Subsequently, the trigger moiety can release an additional RNase H molecule from aptamer-mediated inhibition which, in turn, can release further molecules, thus forming an auto-feedback loop. Active RNase H can also cleave the RNA portion of a DNA/RNA hybrid signalling molecule and produce a detectable signal through the

physical separation of the molecule's fluorophore and quencher.

Figure 8 is a schematic representation of an embodiment of an RNase H-based system with separate linker and multifunctional intermediary/signalling molecule. In its active state following specific hybridisation to a target nucleic acid, the linker is able to release RNase H from aptamer-mediated inhibition. Active RNase H can cleave the RNA portion of the intermediary/signalling molecule and leave the single-stranded trigger moiety exposed. Subsequently, the trigger moiety can release an additional RNase H molecule from aptamer-mediated inhibition which, in turn, can release further molecules, thus forming an auto-feedback loop. Each time an active RNase H molecule digests the RNA portion of the intermediary/signalling molecule, a fluorescent signal is produced by the physical separation of the fluorophore and quencher at the 5' and 3' termini.

Figure 9 is a schematic representation of an embodiment of an RNase H-based system with a multifunctional linker/intermediary/signalling molecule. In its active state following binding to a target nucleic acid, the linker/intermediary/signalling molecule is able to release RNase H from aptamer-mediated inhibition. Active RNase H can cleave the RNA portion of the linker/intermediary and leave the single-stranded trigger moiety exposed. Subsequently, the trigger moiety can release an additional RNase H molecule from aptamer-mediated inhibition which, in turn, can release further molecules, thus forming an auto-feedback loop. Each time an active RNase H molecule digests the RNA portion of the linker/intermediary/signalling molecule, a fluorescent signal is produced by the physical separation of the fluorophore and quencher at the 5' and 3' termini.

Figure 10 shows embodiments of RNase H-inhibiting aptamers and their consensus sequences. Sequences displayed are those of the aptamers following removal of SELEX primer binding sites. Aptamers were able to be placed into two discrete families based on consensus sequences (represented by regions in a particular colour). Aptamers 3.8,

3.9 and 3.22 form one family and aptamers 5.10, 5.2, 5.3, 6.5, 6.6 and 6.13 form another.

Figure 11 shows the predicted tertiary structure of aptamer 3.8.4.1. The presence of multiple G-G di-nucleotides and the lack of an obvious stable secondary structure indicates that aptamer 3.8.4.1 will form a tertiary structure centred on a G-quadruplex. Dots adjacent to some nucleotides indicate the portion of the aptamer complementary to the trigger moiety.

Figure 12 shows the results of trigger-induced release of RNase H from aptamer-mediated inhibition producing a detectable signal. The activity of recombinant *E.coli* RNase H is inhibited when in complex with aptamer 3.8.4.1. The addition of a trigger oligonucleotide, complementary to a portion of aptamer 3.8.4.1, allows the trigger to bind to the aptamer and disrupt its tertiary structure, thus inducing the aptamer to decouple from RNase H and no longer inhibit its activity. Active RNase H is able to digest the RNA portion of a dual-labelled RNA/DNA hybrid signalling molecule, thus rapidly generating a detectable signal.

Figure 13 shows the results of synthetic nucleic acid target (dengue virus)-induced release of RNase H from aptamer-mediated inhibition producing a detectable signal. The linker used contains a moiety complementary to the synthetic nucleic acid target sequence (derived from the genome of dengue virus type 3) and a trigger moiety. Following denaturation and renaturation in the presence of the target oligonucleotide, the trigger moiety in its active state is able to release RNase H from aptamer-mediated inhibition and generate a detectable signal. A degree of background signal is present due to the incomplete renaturation of the linker molecule.

Figure 14 shows the results of synthetic nucleic acid target (NPT)-induced release of RNase H from aptamer-mediated inhibition producing a detectable signal. The linker used contains a moiety complementary to the synthetic nucleic acid target sequence (derived from the neomycin phosphotransferase coding sequence) and a trigger

moiety. Following denaturation and renaturation in the presence of the target oligonucleotide, the trigger moiety in its active state is able to release RNase H from aptamer-mediated inhibition and generate a detectable signal. A degree of background signal is present due to the incomplete renaturation of the linker molecule.

5

Figure 15 shows the results of synthetic nucleic acid target (*Cre*)-induced release of RNase H from aptamer-mediated inhibition producing a detectable signal. The linker used contains a moiety complementary to the synthetic nucleic acid target sequence (derived from the *Cre* recombinase coding sequence) and a trigger moiety. Following
10 denaturation and renaturation in the presence of the target oligonucleotide, the trigger moiety in its active state is able to release RNase H from aptamer-mediated inhibition and generate a detectable signal. A degree of background signal is present due to the incomplete renaturation of the linker molecule.

15 Figure 16 shows the sequence and predicted secondary structure of an embodiment of an auto-feedback loop intermediary. The DNA trigger moiety is shaded in green and is complementary to a portion of the RNase H-inhibiting aptamer 3.8.4.1. When bound to its RNA complement, the trigger moiety is unable to bind to aptamer 3.8.4.1. The RNA
20 portion (shaded orange) forms, when bound to its DNA complement, the substrate for RNase H digestion. Following RNase H digestion, the trigger moiety is able to bind to aptamer 3.8.4.1 and release its bound RNase H molecule from inhibition.

Figure 17 shows the results of a trigger-induced auto-feedback loop using a discrete intermediary molecule and an RNase H/aptamer complex. The reaction was initiated
25 by the addition of a 'trigger' oligonucleotide. The presence of a discrete intermediary molecule resulted in a significant increase in the rate at which a signal was generated compared to the control reaction where no intermediary was added.

Figure 18 shows the results of a target nucleic acid-induced auto-feedback loop using a
30 discrete intermediary molecule and an RNase H/aptamer complex. The reaction was

initiated by the binding of a linker molecule to a synthetic dengue virus type 4-derived oligonucleotide. The presence of discrete intermediary molecules resulted in a significant increase in the rate at which a signal was generated compared to the control reaction where no intermediaries were added. Due to active RNase H present in the
5 'no target' control through incomplete inhibition of RNase H along with incomplete renaturation of all linker molecules, the 'no target' signal was also amplified to a degree.

Figure 19 shows the results of a target nucleic acid-induced auto-feedback loop using a
10 multifunctional intermediary/signalling molecule and an RNase H/aptamer complex. The reaction was initiated by the binding of a linker molecule to a synthetic dengue virus type 4-derived oligonucleotide. The presence of multifunctional signalling/intermediary molecules resulted in a significant increase in the rate at which a signal was generated compared to the control reaction where only single-function
15 signalling molecules were added. Due to active RNase H present in the 'no target' control through incomplete inhibition of RNase H along with incomplete renaturation of all linker molecules, the 'no target' signal was also amplified to a degree.

EXAMPLE 1

Restriction endonuclease-based autofeedback loop system

In this embodiment the nuclease is a restriction endonuclease (e.g. *EcoRI*). Each restriction endonuclease can rapidly and specifically cleave DNA that contains its particular recognition sequence (see Figure 1).

25 The specific hybridisation of the linker and target nucleic acid occurs through heat- or chemically-induced denaturation followed by cooling- or chemically-induced renaturation. The linker is able to hybridise to a target nucleic acid if present in solution via a binder sequence, thus switching to an active state by fully exposing the
30 aptamer-complementary portion of its denatured stem (ie. the trigger nucleotide

sequence). In the absence of the target nucleic acid, the linker reforms a closed stem-loop structure and remains stable in an inactive form. The linker is designed such that the melting temperature of the stem is above the reaction temperature but below that of the hybridised binder/target nucleic acid. In addition, the linker is also designed to remain stably bound to a target sequence if present in solution or stably return to its original conformation following chemical renaturation.

Following the addition of the restriction endonuclease-aptamer complex along with the intermediary and signalling molecules to the prepared linker/nucleic acid mixture, an auto-feedback loop commences if there are active linker molecules present in solution. The active restriction endonuclease molecule/s are then able to cleave multiple intermediary molecules containing the enzyme's recognition sequence.

The intermediary molecule may be an oligonucleotide with a stem/loop hairpin structure. In some embodiments, it performs the intermediary function only. In other embodiments, it has the dual function of acting as a linker nucleotide sequence as well as an intermediary nucleotide sequence. The intermediary possesses a restriction enzyme recognition site and an aptamer-binding trigger sequence. The intermediary is designed such that the melting temperature of the uncleaved stem portion is significantly above the temperature that the assay is conducted at. However, following restriction endonuclease cleavage, the melting temperature of the resultant double-stranded stem portion is below the temperature that the auto-feedback loop reaction is conducted at, thus allowing the trigger portion to melt away from its complement (see Figure 2).

25

When free in solution, the single-stranded trigger portion is able to bind to an aptamer in a restriction endonuclease-aptamer complex and release the restriction endonuclease from inhibition. The generated auto-feedback loop continues until theoretically all restriction endonuclease molecules are released from inhibition. A signal is generated by the specific cleavage of dual-labelled hairpin signalling molecules, such as

30

Molecular Break-Lights (Biggins *et al.*, *Proc Natl Acad Sci USA* 97(25): 13537–13542, 2000). As further restriction endonuclease molecules are released from inhibition, the rate at which a signal is generated rapidly increases. The system may be theoretically able to detect as little as one copy of a target nucleic acid in solution.

5

Using a system where the auto-feedback loop is induced by the addition of a trigger oligonucleotide rather than the presence of a target nucleic acid, a large increase in signal intensity was demonstrated (see Figure 3). The reaction was performed both with and without an auto-feedback loop-generating intermediary. A barely detectable
10 signal was generated in the absence of the intermediary. The presence of the intermediary allowed for rapid signal generation to occur, with all signalling molecules cleaved after a period of approximately 90 minutes. Due to incomplete aptamer-mediated inhibition of *EcoRI*, the intermediary also amplifies the 'no trigger' negative control signal to a lesser extent.

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An example of an auto-feedback loop induced by the binding of a linker to its complementary target nucleic acid is shown in Figure 4. In this example, the functions of the linker and intermediary are performed by separate molecules. The synthetic single-stranded oligonucleotide target is based on a portion of the *Mycobacterium tuberculosis* genome. The presence of the intermediary significantly amplifies the signal
20 induced by the presence of the target sequence compared to the control treatment where no intermediary is present. As with the 'trigger only' example assay, the 'no target' treatment signal is also amplified, albeit to a lesser degree, due to incomplete inhibition of *EcoRI* and, additionally, incomplete re-folding of a small portion of non-target-bound linker molecules.
25

An example of a reaction performed using a single discrete molecule that performs the function of both the linker and intermediary is shown in Figure 5. For comparison, a control linker-only molecule was used. The control linker possessed a nucleotide
30 substitution at the *EcoRI* recognition site, rendering the molecule unable to be cleaved

by *EcoRI* and, therefore, unable to contribute to an auto-feedback loop. Both the linker/intermediary and the control linker were able to produce a detectable signal by binding to the target oligonucleotide and releasing *EcoRI* from aptamer-mediated inhibition. However, the reaction containing the intermediary/linker produced

5 significantly greater signal intensity due to the resultant cleavage of intermediary/linker molecules that were unbound to target oligonucleotides and, accordingly, possessed intact *EcoRI* recognition sites. Continual cleavage of intermediary/linker molecules and the release of further *EcoRI* molecules from aptamer-mediated inhibition resulted in a greater signal intensity for the 'target

10 present' treatment and, to a lesser extent, the 'no target' control.

Materials and Methods

For the biosensor assays, a synthesised version of the strong inhibitor aptamer 3 was

15 used (Sigma-Proligo). Aptamer 3 was identified using the SELEX process (e.g. Tuerk and Gold, *Science* 249(4968): 505–510, 1990). The synthetic aptamer, aptamer 3_ADJ_L, was identical in sequence to aptamer 3 except for the removal of a portion of both primer binding sites. The total length of aptamer 3_ADJ_L was 49 nucleotides (CCGACGAGCAAGTAGCTCCAAGACGAGTTCAACCCAGAATCAGGTCGG; SEQ ID NO: 1).

20

Sequences of other nucleic acid molecules used in these assays were:

- aptamer 3_ADJ_L trigger (GTTGAACTCGTCTTG; SEQ ID NO: 2);
- intermediary (GTTGAACTCGTCTTGAATTCAGTTTTCTGAATTCAACACG GGTTC; SEQ ID NO: 3);
- 25 • tuberculosis linker (GACGTGTTCAACCAGTTTCCCAGGCTTATCCCGAAG TCGGTTGAACTCGTCTTG; SEQ ID NO: 4);
- synthetic tuberculosis target (GCACTTCGGGATAAGCCTGGGAACTG; SEQ ID NO: 5) (Operon)
- linker/intermediary (GTTGAACTCGTCTTGAATTCAGGCCCGGCAGTCTCTCAC
- 30 GAGACCCACCCCTGAATTCAACACGGGTTC; SEQ ID NO: 6);

- linker with modified *EcoRI* recognition site to prevent intermediary function (GTTGAACTCGTCTTGAAATGAGGCCCGGCAGTCTCTCACGAGACCCACCCCTCATTTCAACACGGGTTC; SEQ ID NO: 7) (Operon);
- linker/intermediary target (GGTGGGGTCTCGTGAGAGACTGCCGGGC; SEQ ID NO: 8); and
- Molecular Break-Light signalling molecule ([Fluorescein]GAGAATTCAGTTTCTGAATTCTC[Dabcyl-Q]; SEQ ID NO: 9) (Operon).

All oligonucleotides were synthesised by Sigma Aldrich, formally Sigma-Proligo, unless otherwise indicated.

Trigger-induced auto-feedback loop

Prior to carrying out the assay, aptamer 3_ADJ_L (SEQ ID NO: 1) was bound to *EcoRI* at a ratio of approximately 150 ng of aptamer to 20 units of *EcoRI* (New-England Biolabs) in a solution containing 1 µg/µl BSA (New-England Biolabs). Binding took place at 37°C for 45 minutes.

The final concentration of each component of the *EcoRI* trigger-induced auto-feedback loop assay was: Molecular Break-Light signalling molecule (266 nM) (SEQ ID NO: 9); aptamer 3_ADJ_L (500 nM) (SEQ ID NO: 1); *EcoRI* (20 units); 1.5x NEB *EcoRI* buffer (150 mM Tris-HCl; 75 mM NaCl; 15 mM MgCl₂; 0.0375% Triton X-100; pH 7.5 @ 25°C); BSA (1 µg/µl); intermediary (500 nM) (SEQ ID NO: 3); and trigger (250 nM) (SEQ ID NO: 2). Reactions were made up to 20 µl with deionised water. Two replicates for each treatment were analysed. Analysis was carried out on a Corbett RotorGene RG-3000 set to a constant temperature of 30°C. Fluorescence was measured on the FAM channel at intervals of one minute.

Nucleic acid target-induced auto-feedback loop with discrete linker and intermediary

Prior to carrying out the assay, aptamer 3_ADJ_L (SEQ ID NO: 1) was bound to *EcoRI* at a ratio of approximately 42.5 ng of aptamer to 5 units of *EcoRI* (New-England Biolabs) in a solution containing 1.4 µg/µl BSA (New-England Biolabs). Binding took place at 37°C for 40 minutes.

A solution comprised of 1.9 µg linker (SEQ ID NO: 4) and, if present, 0.9 µg of tuberculosis target nucleic acid (SEQ ID NO: 5) was denatured with 9 µl 150 mM NaOH for 60 seconds then renatured by neutralisation with 6 µl 200 mM HCl and 1.1 µl 1 M Tris-HCl (pH 8.0). The final volume of the reaction was 18.4 µl. 0.25 µl of this solution was added to the final biosensor reaction.

The final concentration of each component of the *EcoRI* nucleic acid target-induced auto-feedback loop assay was: Molecular Break-Light signalling molecule (67 nM) (SEQ ID NO: 9); aptamer 3_ADJ_L (142 nM) (SEQ ID NO: 1); *EcoRI* (5 units); BSA (1 µg/µl); 1.5x NEB *EcoRI* buffer (150 mM Tris-HCl; 75 mM NaCl; 15 mM MgCl₂; 0.0375% Triton X-100; pH 7.5 @ 25°C); intermediary (125 nM) (SEQ ID NO: 3); linker (75 nM) (SEQ ID NO: 4); and tuberculosis target (75 nM) (SEQ ID NO: 5). Reactions were made up to 20 µl with deionised water. Two replicates for each treatment were analysed. Analysis was carried out on a Corbett RotorGene RG-3000 set to a constant temperature of 30°C. Fluorescence was measured on the FAM channel at intervals of one minute.

Nucleic acid target-induced auto-feedback loop with a combined linker and intermediary molecule

Prior to carrying out the assay, aptamer 3_ADJ_L (SEQ ID NO: 1) was bound to *EcoRI* at a ratio of approximately 37.5 ng of aptamer to 5 units of *EcoRI* (New-England Biolabs) in a solution containing 1.5 µg/µl BSA (New-England Biolabs). Binding took

place at 37°C for 40 minutes.

A solution comprised of 2.4 µg linker/intermediary (SEQ ID NO: 6) and, if present, 1 µg of target nucleic acid (SEQ ID NO: 8) was denatured with 9 µl 150 mM NaOH for 60 seconds then renatured by neutralisation with 6 µl 200 mM HCl and 1.1 µl 1 M Tris-HCl (pH 8.0). The final volume of the reaction was 18.4 µl. 1 µl of this solution was added to the final biosensor reaction.

The final concentration of each component of the *EcoRI* nucleic acid target-induced auto-feedback loop assay was: Molecular Break-light Signalling molecule (67 nM) (SEQ ID NO: 9); aptamer 3_ADJ_L (125 nM) (SEQ ID NO: 1); *EcoRI* (5 units); BSA (1 µg/µl); buffer (150 mM Tris-HCl; 75 mM NaCl; 10 mM MgCl₂; 0.0375% Triton X-100; pH 7.5 @ 25°C); linker/intermediary (SEQ ID NO: 6) or control linker-only molecule (SEQ ID NO: 7) (300 nM) ; and target (300 nM) (SEQ ID NO: 8). Reactions were made up to 20 µl with deionised water. Two replicates for each treatment were analysed. Analysis was carried out on a Corbett RotorGene RG-3000 set to a constant temperature of 30°C. Fluorescence was measured on the FAM channel at intervals of one minute.

EXAMPLE 2

RNase H-based autofeedback loop system

The RNase H system is based on aptamer-mediated inhibition of RNase H that, similarly to the *EcoRI* system, can be released by the binding of a specific trigger oligonucleotide. There are numerous embodiments of an RNase H system. The linker, intermediary and signalling molecules may be unique, or may be multifunctional molecules which carry out two or all the roles of these individual molecules, for examples, see Figures 6, 7, 8 and 9.

The linker molecule in this example of the RNase H-based system acts in the same fashion as its counterpart in the *EcoRI*-based system. It has a stem/loop hairpin

secondary structure in its inactive state. The loop portion is complementary to the target nucleic acid and one complement of the stem portion is complementary to a segment of the RNase H-inhibiting aptamer used in the reaction. In its active state following hybridisation to a target nucleic acid through denaturation and renaturation, the single-stranded DNA trigger moiety can bind to an aptamer in an aptamer/RNase H complex and release RNase H from inhibition. Each strand of the stem can be composed of solely of DNA or solely of RNA if the linker molecule has a single function only. If the linker is multifunctional and operates as an intermediary and/or signalling molecule, the stem is a DNA/RNA hybrid, with the trigger moiety composed of DNA and its complement composed of RNA. The linker is designed such that the melting temperature of the stem is above the reaction temperature but below that of the hybridised loop/target nucleic acid.

The intermediary and signalling molecules are DNA/RNA hybrids. Active RNase H can digest the RNA portion of a DNA/RNA hybrid and leave the DNA portion intact. In the case of the molecule acting as an intermediary, the remaining single-stranded DNA portion forms the trigger moiety, which is able to bind to an aptamer in an aptamer/RNase H complex and release RNase H from inhibition in the same fashion as the linker. The continual RNase H-mediated cleavage of intermediaries and the release of additional RNase H molecules from inhibition form the basis of an auto-feedback loop.

The DNA/RNA hybrid signalling molecule is labelled at one terminus with a fluorophore and at the other a quencher, and forms a hairpin stem/loop secondary structure at reaction temperatures. To prevent signal quenching via hybridisation of the DNA trigger portion of a dual-function intermediary/signalling molecule to an aptamer, the fluorophore may be situated on the RNA moiety. When the molecule is intact, the fluorophore's signal is strongly quenched by the quencher through Förster resonance energy transfer (FRET). Upon physical separation by means of RNase H cleavage, a detectable fluorescent signal is generated.

- A modified version of the SELEX protocol (e.g. Tuerk and Gold, *Science* 249(4968): 505–510, 1990) was used to select an appropriate aptamer that strongly inhibited the activity of RNase H whilst remaining amenable to trigger-mediated release. Identified RNase H-inhibiting aptamer sequences grouped into families by consensus are shown in Figure 10. The GGGTGGGT consensus nucleotide sequence motif was designated as SEQ ID NO: 10, while the GGGGGGCATCG consensus nucleotide sequence motif was designated as SEQ ID NO: 11.
- 10 The aptamer selected for use in the RNase H biosensor (aptamer 3.8; SEQ ID NO: 13) was truncated by removal of terminal nucleotides to further improve its inhibition ability (aptamer 3.8.4.1; SEQ ID NO: 12). Due to the presence of multiple G·G dinucleotide motifs, a tertiary structure based on a G-quartet arrangement is likely. To release RNase H from aptamer 3.8.4.1-mediated inhibition, numerous complementary trigger oligonucleotides were designed and tested. The best performing trigger molecule was able to rapidly release RNase H from inhibition. The trigger and aptamer/RNase H complex constitute the switching mechanism that forms the basis of the RNase H biosensor (see Figures 11 and 12).
- 20 Specific linker molecules containing the identified trigger were developed to detect the presence of synthetic dengue virus types 3 and 4, neomycin phosphotransferase and *Cre* recombinase nucleic acid sequences in solution. The RNase H biosensor was able to indicate the presence or absence of each target sequence (see Figures 13, 14 and 15).
- 25 Initially, single-function intermediaries were developed for the RNase H biosensor. For the best performing intermediary, the trigger moiety formed one strand of the stem and, additionally, the loop, with the complementary RNA strand situated at the 3' end of the molecule (Figure 16).
- 30 A rapid increase in signal intensity via a trigger-induced auto-feedback loop generated

by a single-function intermediary is shown in Figure 17. A barely detectable signal was produced in the control reaction lacking an auto-feedback loop-generating intermediary. Due to incomplete aptamer-mediated inhibition of RNase H, the intermediary also amplified the 'no trigger' negative control signal to a lesser degree.

5

Examples of an auto-feedback loop induced by the presence of a synthetic target oligonucleotide are shown in Figures 18 and 19. The synthetic targets are based on portions of the dengue virus type 4 genome. The presence of the intermediary significantly amplified the signal induced by the presence of the target sequence compared to the control treatment where no intermediary was present.

10

Materials and Methods

For each biosensor assay, aptamer 3.8.4.1 (AGGGAATGCTAGGGGGGGTGGGT; SEQ ID NO: 12) was used. Aptamer 3.8.4.1 (was derived from aptamer 3.8 (CGAAGGGAATGCTAGGGGGGGTGGGTGTGA; SEQ ID NO: 13), identified using a modified semi-automated SELEX protocol based on Wochner *et al.* (*Biotechniques* 3(43): 344-353, 2007).

15

20 Other RNase-H inhibiting aptamers identified include:

- Aptamer 3.9 (CACAGGGAGGGGTGCTAGGGTGGGTTATGT; SEQ ID NO: 14);
- Aptamer 3.22 (CGGGGTGGGTGTGCCAGGGCGGGATGCTCG; SEQ ID NO: 15);
- Aptamer 5.10 (GCGGGGGGGGCATCGCGCTCGGGGAGGGGCTACATGAC; SEQ ID NO: 16);
- Aptamer 5.2 (GGGGGGCATCGCGGATCATGAGCGGGAGGAACCTAAATAC; SEQ ID NO: 17);
- Aptamer 5.3 (GGGACGTGGGGGGCATCGCGTCCGCCTCATGG; SEQ ID NO: 18);
- Aptamer 6.5 (GGGGGGGCATCGTCGATTATGTAAGGGGCC; SEQ ID NO: 19);
- Aptamer 6.6 (GGGGGGGCATCGTCGGGGGGTGCCTGGCC; SEQ ID NO: 20) and
- Aptamer 6.13 (GGGGGGGCATCGTCGAGACGGGGAGGCCCT; SEQ ID NO: 21)

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Other sequences used in the RNase H biosensor assays include:

- Aptamer 3.8.4.1 trigger (CCCACCCCCCTAGCAT; SEQ ID NO: 22);
- the dengue type 3 linker (ACCCCCCTAGCATACGCACCCAAACCTGGAT
5 GTCGATGCTAGG; SEQ ID NO: 23);
- the synthetic dengue type 3 target (CGACATCCAGGTTTGGGTGCGT; SEQ ID NO: 24);
- the dengue type 4 linker 1 (ATGCTAGGGCGGGTGGGCATGCACCCA
GAGCGGAGAACGGCCACCCCCCTAGCAT; SEQ ID NO: 25);
- 10 • the dengue type 4 linker 2 (ATGCAAGGGCGGGTGGGGCCAAG
AGCGCGAATCCTGGGTCCCCACCCCCCTAGCAT; SEQ ID NO: 34);
- the synthetic dengue type 4 target 1 (CCGTTCTCCGCTCTGGGTGCATG; SEQ ID
NO: 26);
- the synthetic dengue type 4 target 2 (AACCCAGGATTCGCGCTCTTGGC; SEQ ID
15 NO: 35);
- the neomycin phosphotransferase linker (ACCCCCCTAGCATGCGAT
GCGCTGCGAATCGGGAGCATGCTAGG; SEQ ID NO: 27);
- the synthetic neomycin phosphotransferase target (GCTCCCGATTTCGC
AGCGCATCGC; SEQ ID NO: 28);
- 20 • the *Cre* recombinase linker (ACCCCCCTAGCATCGGTTCGTGG
GCGGCATGGTGCAATGCTAGGGG; SEQ ID NO: 29);
- the synthetic *Cre* recombinase target (TGCACCATGCCGCCACGACCG; SEQ ID
NO: 30);
- the discrete intermediary (ACCCCCCTAGCATUrArGrGrGrGrGrG; SEQ ID NO:
25 31) where ribonucleotides other than uracil are indicated by a preceding 'r';
- the discrete molecular break-light signalling molecule 1
([Fluorescein]GTTGAACTCGTCTTGTTrCrArArGrArCrGrArGUUrCrArA
rC[Dabcyl-Q]; SEQ ID NO: 32) - where ribonucleotides other than uracil are
indicated by a preceding 'r';
- 30 • the discrete molecular break-light signalling molecule 2 ([6-FAM]GAGA

ATTTCAGTTTTTrCUrGrArAUUrCUrC[Dabcyl-Q]; SEQ ID NO: 33) - where ribonucleotides other than uracil are indicated by a preceding 'r'; and

- the multifunctional intermediary/signalling molecule ([6-FAM]ACCCCCCCTAGCATUrArGrGrGrGrGrG[Dabcyl-Q]) (SEQ ID NO: 36).

5

All oligonucleotides were synthesised by Sigma Aldrich, formally Sigma-Prologo, except for the multifunctional intermediary/signalling molecule, which was synthesised by Integrated DNA Technologies.

- 10 For recombinant RNase H synthesis, a plasmid containing an artificial gene construct based on the amino acid sequence of *E.coli* RNase H but with optimised codon usage (Mr Gene GmbH) was used as a template for PCR. PCR was performed using the QIAGEN EasyXpress Linear Template Kit Plus, which allowed for the addition of a C-terminal or N-terminal 6xHis tag. RNase H was synthesised with the Expressway™
- 15 Cell-Free *E.coli* expression system (Invitrogen) and purified with Ni-NTA Magnetic agarose beads (QIAGEN). Linear template synthesis, cell-free expression and purification were all performed using the manufacturer's protocol.

- Once synthesised RNase H was dialysed into 1x RNase H storage buffer (20mM Tris-HCl; 100mM KCl; 10mM MgCl₂; 0.1mM DTT; 0.1mM EDTA; 50% Glycerol; pH 7.5 @
- 20 25°C). Due to dialysis and addition of buffer components RNase H was diluted during the process approximately 16-fold for the C-terminal 6xHis tag recombinant and 4-fold for the N-terminal 6xHis tag recombinant. Storage thereafter was at -20°C.

- 25 *Trigger-induced release of RNase H from aptamer-mediated inhibition and signal generation*

- Prior to carrying out the assay, aptamer 3.8.4.1 (SEQ ID NO: 12) was heat-denatured at 95°C for 2 minutes and subsequently cooled on ice. Recombinant N-terminal 6xHis-tagged RNase H was diluted 10 times by volume with deionised water. The prepared
- 30 aptamer was bound to RNase H at a ratio of approximately 90 ng of aptamer to 0.1 µl

of diluted RNase H in a solution containing 1.1 µg/µl BSA (New-England Biolabs). Binding took place at room temperature for 45 minutes.

The final concentration of each component of the RNase H trigger-induced biosensor assay was: signalling molecule (55 nM) (SEQ ID NO: 32); aptamer 3.8.4.1 (450 nM) (SEQ ID NO: 12); diluted RNase H (0.1 µl); BSA (1 µg/µl); buffer (50 mM Tris-HCl; 3 mM MgCl₂; 0.5 mM KCl; 100 mM DTT) and trigger (5 µM) (SEQ ID NO: 22). Reactions were made up to 20 µl with deionised water. Two replicates for each treatment were analysed. Analysis was carried out on a Corbett RotorGene RG-3000 set to a constant temperature of 30°C. Fluorescence was measured on the FAM channel at intervals of one minute.

Nucleic acid target-induced release of RNase H from aptamer-mediated inhibition and signal generation

Prior to carrying out the dengue linker and target assay, aptamer 3.8.4.1 (SEQ ID NO: 12) was heat-denatured at 95°C for 2 minutes and subsequently cooled on ice. Recombinant C-terminal 6xHis-tagged RNase H was diluted 100 times by volume with deionised water. The prepared aptamer was bound to RNase H at a ratio of approximately 54 ng of aptamer to 0.2 µl of RNase H in a solution containing 1.1 µg/µl BSA (New-England Biolabs). For the Cre recombinase and neomycin phosphotransferase assays, aptamer 3.8.4.1 (SEQ ID NO: 12) was bound to diluted RNase H at a ratio of approximately 66 ng of aptamer to 0.2 µl of RNase H in a solution containing 1.1 µg/µl BSA (New-England Biolabs). Binding took place at room temperature for 40 minutes.

A solution comprised of 1.4 µg linker (dengue 3 - SEQ ID NO: 23; NPT - SEQ ID NO: 27; or Cre - SEQ ID NO: 29) and 0.7 µg of the appropriate target nucleic acid (dengue 3 - SEQ ID NO: 24; NPT - SEQ ID NO: 28; or Cre - SEQ ID NO: 30) was denatured with 1.4 µl 1 M NaOH for 60 seconds then renatured by neutralisation with 6 µl 200 mM HCl

and 1.1 µl 1 M Tris-HCl (pH 8.0). The final volume of the reaction was 12 µl. 1 µl of this solution was added to the final biosensor reaction.

The final concentration of each component of the RNase H nucleic acid-induced biosensor assay was: signalling molecule (55 nM) (SEQ ID NO: 33); aptamer 3.8.4.1 (SEQ ID NO: 12) (375 nM for the dengue assay and 450 nM for the Cre and NPT assays); diluted RNase H (0.2 µl); BSA (1 µg/µl); buffer (0.5mM KCl; 50mM Tris; 3mM MgCl₂; 10mM DTT; pH 8.3 @ 25°C); linker (415 nM); and nucleic acid target (415 nM). Reactions were made up to 20 µl with deionised water. Two replicates for each treatment were analysed. Analysis was carried out on a Corbett RotorGene RG-3000 set to a constant temperature of 30°C. Fluorescence was measured on the FAM channel at intervals of one minute.

Trigger-induced auto-feedback loop

Prior to carrying out the trigger-induced auto-feedback loop assay, aptamer 3.8.4.1 (SEQ ID NO: 12) was heat-denatured at 95°C for 2 minutes and subsequently cooled on ice. Recombinant C-terminal 6xHis-tagged RNase H was diluted 100 times by volume with deionised water. The prepared aptamer was bound to RNase H at a ratio of approximately 33 ng of aptamer to 0.2 µl of diluted RNase H in a solution containing 1.2 µg/µl BSA (New-England Biolabs). Binding took place at room temperature for 15 minutes.

The final concentration of each component of the trigger-induced auto-feedback loop assay was: signalling molecule (55 nM) (SEQ ID NO: 33); aptamer 3.8.4.1 (225nM) (SEQ ID NO: 12); diluted RNase H (0.2 µl); BSA (1µg/µl); buffer (3.5mM KCl; 50mM Tris; 3mM MgCl₂; 10mM DTT; pH 8.3 @ 25°C); trigger (110 uM) (SEQ ID NO: 22) and intermediary (310 nM) (SEQ ID NO: 31). Reactions were made up to 20 µl with deionised water. Two replicates for each treatment were analysed. Analysis was carried out on a Corbett RotorGene RG-3000 set to a constant temperature of 30°C.

Fluorescence was measured on the FAM channel at intervals of one minute.

Nucleic acid target-induced auto-feedback loop with discrete linker and intermediary

5 Prior to carrying out the nucleic acid-induced auto-feedback loop assay, aptamer 3.8.4.1 (SEQ ID NO: 12) and the intermediary (SEQ ID NO: 31) were separately heat-denatured at 95°C for 2 minutes and subsequently cooled on ice. Recombinant C-terminal 6xHis-tagged RNase H was diluted 100 times by volume with deionised water. The prepared aptamer was bound to RNase H at a ratio of approximately 33 ng
10 of aptamer to 0.3 µl of RNase H in a solution containing 1.3 µg/µl BSA (New-England Biolabs). Binding took place at room temperature for 20 minutes.

A solution comprised of 2.6 µg dengue 4 linker 1 (SEQ ID NO: 25) and 0.7 µg of dengue 4 target 1 nucleic acid (SEQ ID NO: 26) was denatured with 2.7 µl 1 M NaOH
15 for 60 seconds then renatured by neutralisation with 12 µl 200 mM HCl and 2.2 µl 1 M Tris-HCL (pH 8.0). The final volume of the reaction was 24 µl. 1 µl of this solution was added to the final biosensor reaction.

The final concentration of each component of the nucleic acid-induced auto-feedback
20 loop assay was: signalling molecule (82.5 nM) (SEQ ID NO: 33); aptamer 3.8.4.1 (225nM) (SEQ ID NO: 12); diluted RNase H (0.3 µl); buffer (1.5mM KCl; 50mM Tris; 3mM MgCl₂; 10mM DTT; pH 8.3 @ 25°C); linker (310 uM) (SEQ ID NO: 25); target nucleic acid (210 nM) (SEQ ID NO: 26); and intermediary (540 nM) (SEQ ID NO: 31). Reactions were made up to 20 µl with deionised water. Four replicates for each
25 treatment were analysed. Analysis was carried out on a Corbett RotorGene RG-3000 set to a constant temperature of 30°C. Fluorescence was measured on the FAM channel at intervals of one minute.

Nucleic acid target-induced auto-feedback loop with a multifunctional intermediary/signalling molecule:

Prior to carrying out the nucleic acid-induced auto-feedback loop assay, aptamer
5 3.8.4.1 (SEQ ID NO: 12) and the intermediary/signalling molecule (SEQ ID NO: 36)
were separately heat-denatured at 95°C for 2 minutes and subsequently cooled on ice.
Recombinant C-terminal 6xHis-tagged RNase H was diluted 100 times by volume with
deionised water. The prepared aptamer was bound to RNase H at a ratio of
approximately 33 ng of aptamer to 0.3 µl of RNase H in a solution containing 1.3 µg/µl
10 BSA (New-England Biolabs). Binding took place at room temperature for 20 minutes.

A solution comprised of 1.8 µg dengue 4 linker 2 (SEQ ID NO: 34) and 0.6 µg of
dengue type 4 target 2 nucleic acid (SEQ ID NO: 35) was buffered with 1.5mM KCl,
50mM Tris_HCl, 3mM MgCl₂, and 10mM DTT. The final volume of the reaction was 25
15 µl. The solution was heated to 95°C for two minutes then cooled on ice. 1 µl of this
solution was added to the final biosensor reaction.

The final concentration of each component of the nucleic acid-induced auto-feedback
loop assay was: aptamer 3.8.4.1 (225nM) (SEQ ID NO: 12); diluted RNase H (0.3 µl);
20 buffer (1.5mM KCl; 50mM Tris; 3mM MgCl₂; 10mM DTT; pH 8.3 @ 25°C); linker (200
uM) (SEQ ID NO: 34); target nucleic acid (200 nM) (SEQ ID NO: 35); and
intermediary/signalling molecule (538 nM) (SEQ ID NO: 36). Reactions were made up
to 20 µl with deionised water. Two replicates for each treatment were analysed.
Analysis was carried out on a Corbett RotorGene RG-3000 set to a constant
25 temperature of 30°C. Fluorescence was measured on the FAM channel at intervals of
two minutes.

Those skilled in the art will appreciate that the invention described herein is
susceptible to variations and modifications other than those specifically described. It is
30 to be understood that the invention includes all such variations and modifications. The

invention also includes all of the steps, features, compositions and compounds referred to, or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features.

- 5 Also, it must be noted that, as used herein, the singular forms “a”, “an” and “the” include plural aspects unless the context already dictates otherwise.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS

1. A method for detecting a target in a sample, the method comprising:

5 providing a population of nuclease-aptamer complexes, each complex comprising a nuclease bound to an aptamer, wherein binding of the aptamer to the nuclease inhibits the activity of the nuclease;

providing an intermediary nucleotide sequence comprising: (i) a nucleotide sequence which is digestible by a nuclease, and (ii) a trigger sequence which can hybridise with an aptamer in a nuclease-aptamer complex when the intermediary
10 nucleotide sequence is digested by a nuclease, wherein hybridisation between the trigger sequence and an aptamer in a nuclease-aptamer complex reduces inhibition of the nuclease by the aptamer; and

applying the population of nuclease-aptamer complexes and the intermediary nucleotide sequence to the sample under conditions such that the presence of the target
15 in the sample directly or indirectly reduces inhibition of a nuclease by an aptamer in a first nuclease-aptamer complex in the population;

wherein a nuclease having reduced inhibition by an aptamer digests an intermediary nucleotide sequence such that hybridisation occurs between a trigger sequence and an aptamer in a second nuclease aptamer complex in the population to
20 reduce inhibition of the second nuclease;

detecting nuclease activity in the sample, wherein increased nuclease activity is indicative of the presence of the target in the sample.

2. The method of claim 1 wherein the method further comprises:

25 providing a linker nucleotide sequence comprising: (i) a binder sequence which can associate with a target if it is present in the sample, and (ii) a trigger sequence which can hybridise with an aptamer in a nuclease-aptamer complex when the binder sequence is associated with the target, wherein hybridisation between the trigger sequence and the aptamer reduces inhibition of the nuclease in the nuclease-aptamer
30 complex by the aptamer; and

applying the linker nucleotide sequence to the sample;

wherein the presence of the target in the sample reduces inhibition of a nuclease via association of the binder sequence with the target which allows hybridisation between the trigger nucleotide sequence and an aptamer in a first
5 nuclease aptamer complex to thus modify, reduce or eliminate binding between the aptamer and the nuclease in the first nuclease-aptamer complex.

3. The method of claim 2 wherein the linker nucleotide sequence is comprised within a nucleic acid having a stem-loop secondary structure and all or part of the
10 binder sequence is comprised within a loop of the stem-loop structure and all or part of the trigger sequence is comprised within a stem of the stem-loop structure.

4. The method of claim 2 or 3 wherein the intermediary nucleotide sequence and the linker nucleotide sequence are comprised within one contiguous nucleic acid
15 molecule.

5. The method of claims 3 and 4 wherein all or part of the intermediary nucleotide sequence is comprised within a stem of the stem-loop structure.

20 6. The method of claim 1 wherein the presence of the target in the sample directly or indirectly reduces inhibition of a nuclease in a first nuclease-aptamer complex via hybridisation of the aptamer in the first nuclease-aptamer complex to all or part of the target to thus modify, reduce or eliminate binding between the aptamer and a nuclease in the first nuclease-aptamer complex.

25

7. The method of any one of claims 1 to 6 wherein the nuclease is a DNase.

8. The method of claim 7 wherein the nuclease is a restriction endonuclease.

30 9. The method of claim 8 wherein the restriction endonuclease is *EcoRI*.

10. The method of any one of claims 1 to 9 wherein the aptamer comprises the nucleotide sequence set forth in SEQ ID NO: 1 or a variant of said aptamer which retains the ability to bind to and inhibit the activity of *EcoRI*.

5

11. The method of any one of claims 1 to 10 wherein the trigger sequence comprises the nucleotide sequence set forth in SEQ ID NO: 2.

12. The method of any one of claims 1 to 11 wherein the intermediary nucleotide
10 sequence comprises the nucleotide sequence set forth in SEQ ID NO: 3.

13. The method of any one of claims 1 to 6 wherein the nuclease is an RNase.

14. The method of claim 13 wherein the RNase is an RNase H.
15

15. The method of claim 14 wherein the aptamer comprises the nucleotide sequence motif set forth in SEQ ID NO: 10.

16. The method of claim 15 wherein the aptamer comprises the nucleotide
20 sequence set forth in SEQ ID NO: 12; or a variant of said aptamer which retains the ability to bind to and inhibit the activity of RNase H.

17. The method of claim 15 wherein the aptamer comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 13, SEQ ID NO: 14 and/or SEQ ID
25 NO: 15; or a variant of said aptamer which retains the ability to bind to and inhibit the activity of RNase H.

18. The method of claim 14 wherein the aptamer comprises the nucleotide sequence motif set forth in SEQ ID NO: 11.

30

19. The method of claim 18 wherein the aptamer comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20 and/or SEQ ID NO: 21; or a variant of said aptamer which retains the ability to bind to and inhibit the activity of RNase H.

5

20. The method of any one of claims 14 to 19 wherein the trigger sequence comprises the nucleotide sequence set forth in SEQ ID NO: 22.

21. The method of any one of claims 14 to 20 wherein the intermediary nucleotide
10 sequence comprises the nucleotide sequence set forth in SEQ ID NO: 31.

22. The method of any one of claims 1 to 21 wherein the activity of the nuclease is determined by the rate or extent of digestion of a reporter nucleotide sequence.

15 23. The method of claim 22 wherein a fluorophore is bound to the reporter nucleotide sequence and a quencher, which quenches the fluorescence of the fluorophore, is also bound to the reporter nucleotide sequence, wherein digestion of all or part of the reporter nucleotide sequence reduces or eliminates the quenching of the fluorophore by the quencher.

20

24. The method of claim 22 or 23 wherein the reporter nucleotide sequence comprises DNA or an analog thereof.

25 25. The method of claim 22 or 23 wherein the reporter nucleotide sequence comprises RNA or an analog thereof.

26. The method of any one of claims 22 to 25 wherein the reporter nucleotide sequence comprises both DNA or an analog thereof and RNA or an analog thereof.

30 27. The method of claim 26 wherein the fluorophore is bound to an RNA portion of

the reporter nucleotide sequence and the quencher is bound to a DNA portion of the reporter nucleotide sequence or, alternatively, the fluorophore is bound to a DNA portion of the reporter nucleotide sequence and the quencher is bound to an RNA portion of the reporter nucleotide sequence.

5

28. The method of any one of claims 22 to 27 wherein the reporter nucleotide sequence is comprised within a Molecular Break-Light nucleic acid molecule.

29. The method of any one of claims 22 to 28 wherein the reporter nucleotide
10 sequence is comprised within the same nucleic acid molecule as the intermediary nucleotide sequence and/or the linker nucleotide sequence.

30. The method of any one of claims 1 to 29 wherein the target is a nucleic acid.

15 31. The method of any one of claims 1 to 29 wherein the target is a polypeptide.

32. An isolated nucleic acid comprising an intermediary nucleotide sequence the intermediary nucleotide sequence comprising: (i) a nucleotide sequence which is digestible by a nuclease, and (ii) a trigger sequence which can hybridise with an
20 aptamer in a nuclease-aptamer complex to reduce inhibition of the nuclease in the nuclease-aptamer complex by the aptamer when the intermediary nucleotide sequence is digested by a nuclease.

33. The isolated nucleic acid of claim 32 wherein the nucleic acid further comprises
25 a binder sequence which can associate with a target.

34. The isolated nucleic acid of claim 33 wherein the binder sequence can associate with a nucleic acid target.

30 35. The isolated nucleic acid of claim 33 wherein the binder sequence can associate

with a polypeptide target.

36. The isolated nucleic acid molecule of any one of claims 32 to 35 wherein the isolated nucleic acid comprises a stem-loop secondary structure and all or part of the intermediary nucleotide sequence is comprised within a stem of the stem-loop structure.

37. The isolated nucleic acid molecule of any one of claims 33 to 35 and 36 wherein all or part of the binder sequence is comprised within a loop of the stem-loop structure.

38. The isolated nucleic acid of any one of claims 32 to 37 wherein complete or partial digestion of the nucleic acid by a nuclease is detectable.

39. The isolated nucleic acid of claim 38 wherein the isolated nucleic acid comprises a fluorophore and a quencher that quenches the fluorescence of the fluorophore and wherein complete or partial digestion of the nucleic acid reduces or eliminates quenching of the fluorophore by the quencher.

40. The isolated nucleic acid of any one of claims 32 to 39 wherein the isolated nucleic acid comprises DNA or an analog thereof.

41. The isolated nucleic acid of any one of claims 32 to 39 wherein the isolated nucleic acid comprises RNA or an analog thereof.

42. The isolated nucleic acid of any one of claims 32 to 41 wherein the isolated nucleic acid comprises both DNA or an analog thereof and RNA or an analog thereof.

43. The isolated nucleic acid of claim 42 wherein the fluorophore is bound to an RNA portion of the isolated nucleic acid and the quencher is bound to a DNA portion of the isolated nucleic acid or, alternatively, the fluorophore is bound to a DNA portion

of the isolated nucleic acid and the quencher is bound to an RNA portion of the isolated nucleic acid.

44. The isolated nucleic acid of any one of claims 32 to 43 wherein the isolated
5 nucleic acid comprises a trigger sequence comprising the nucleotide sequence set forth in SEQ ID NO: 2.

45. The isolated nucleic acid of any one of claims 32 to 44 wherein the isolated
10 nucleic acid comprises an intermediary nucleotide sequence comprising the nucleotide sequence set forth in SEQ ID NO: 3.

46. The isolated nucleic acid of any one of claims 32 to 43 wherein the isolated
15 nucleic acid comprises a trigger sequence comprising the nucleotide sequence set forth in SEQ ID NO: 22.

47. The isolated nucleic acid of any one of claims 32 to 44 wherein the isolated
nucleic acid comprises an intermediary nucleotide sequence comprising the nucleotide
sequence set forth in SEQ ID NO: 31.

20 48. An isolated nucleic acid according to any one of claims 32 to 47 when used according to the method of any one of claims 1 to 31.

49. A kit for performing the method of any one of claims 1 to 31 the kit comprising
an isolated nucleic acid according to any one of claims 32 to 47.

25

50. A kit according to claim 49 wherein the kit further comprises a population of
nuclease-aptamer complexes.

51. An RNase H-binding aptamer wherein binding of the aptamer to the RNase H
30 inhibits the activity of the RNase H.

52. The RNase H-binding aptamer of claim 51 wherein the aptamer comprises the nucleotide sequence motif set forth in SEQ ID NO: 10.

5 53. The RNase H-binding aptamer of claim 52 wherein the aptamer comprises the nucleotide sequence set forth in SEQ ID NO: 12; or a variant of said aptamer which retains the ability to bind to and inhibit the activity of RNase H.

54. The RNase H-binding aptamer of claim 52 wherein the aptamer comprises a
10 nucleotide sequence selected from the group consisting of SEQ ID NO: 13, SEQ ID NO: 14 and/or SEQ ID NO: 15; or a variant of said aptamer which retains the ability to bind to and inhibit the activity of RNase H.

55. The RNase H-binding aptamer of claim 51 wherein the aptamer comprises the
15 nucleotide sequence motif set forth in SEQ ID NO: 11.

56. The RNase H-binding aptamer of claim 55 wherein the aptamer comprises a
nucleotide sequence selected from the group consisting of SEQ ID NO: 16, SEQ ID NO:
17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20 and/or SEQ ID NO: 21; or a variant
20 of said aptamer which retains the ability to bind to and inhibit the activity of RNase H.

57. An RNase H-aptamer complex comprising RNase H bound to an aptamer,
wherein binding of the aptamer to the RNase H inhibits the activity of the RNase H in
the RNase H-aptamer complex.

25

58. The RNase H-aptamer complex of claim 57 wherein the aptamer is an aptamer
according to any one of claims 51 to 56.

59. The method of any one of claims 14 to 31 wherein the nuclease-aptamer
30 complex is an RNase H-aptamer complex according to claim 57 or 58.

60. A kit for performing the method of any one of claims 14 to 31 the kit comprising an RNase H-aptamer complex according to claim 57 or 58.

FIGURE 1

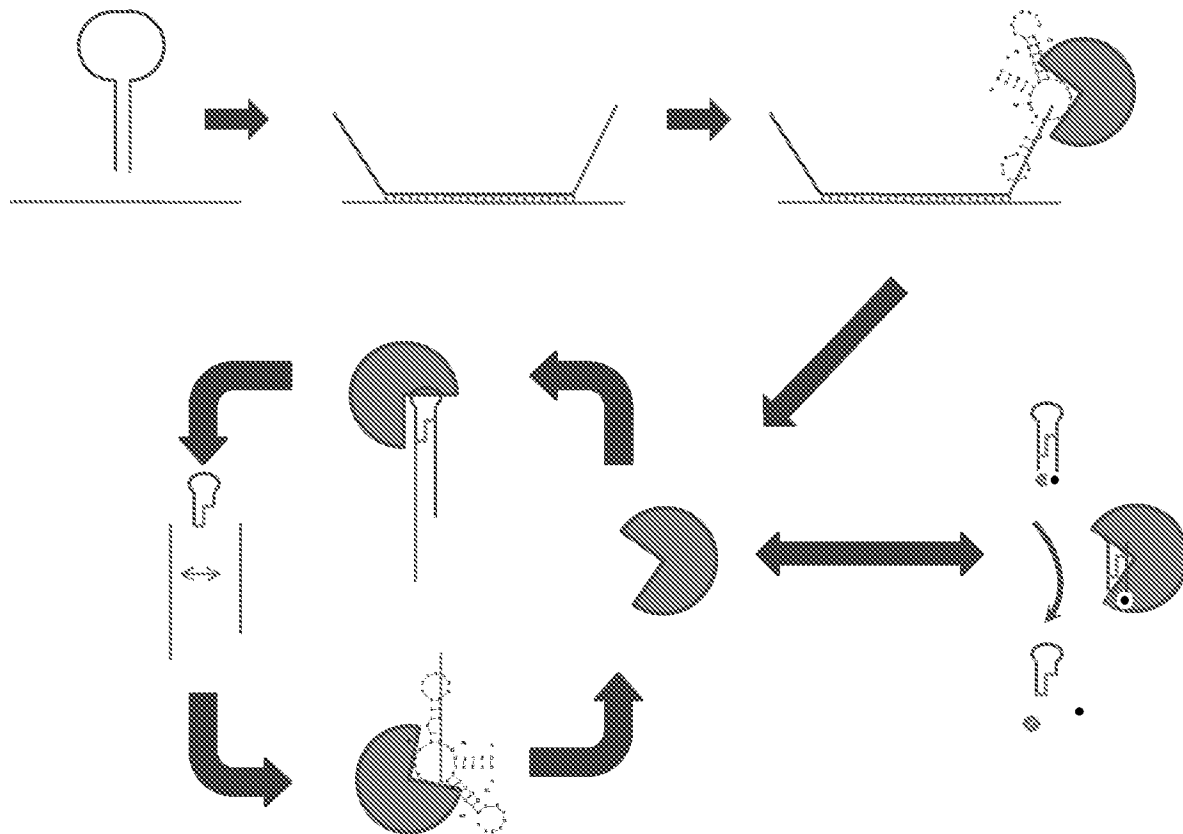
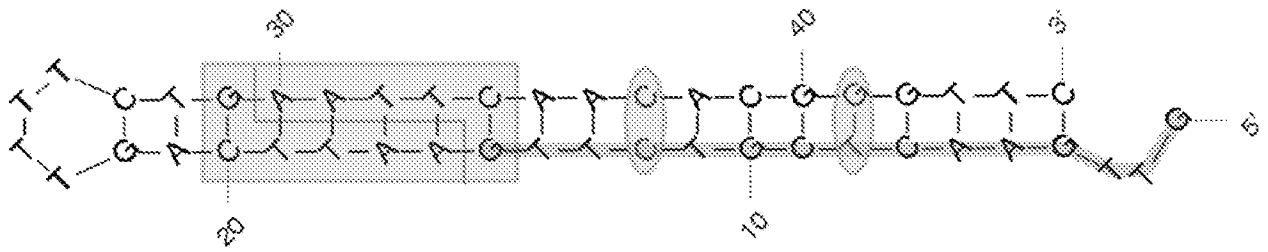
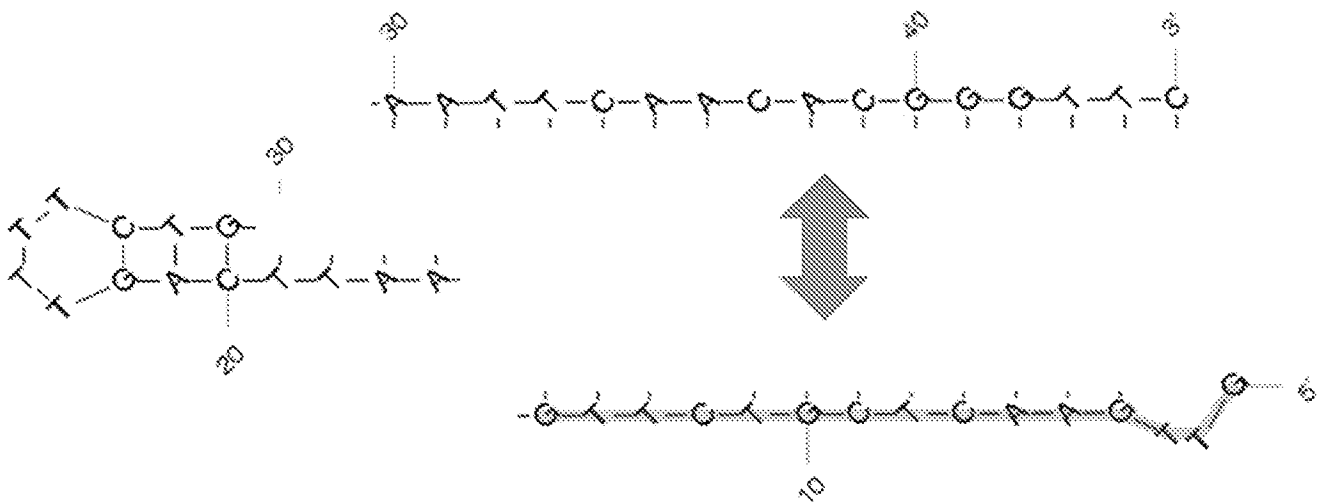


FIGURE 2

A



B



C

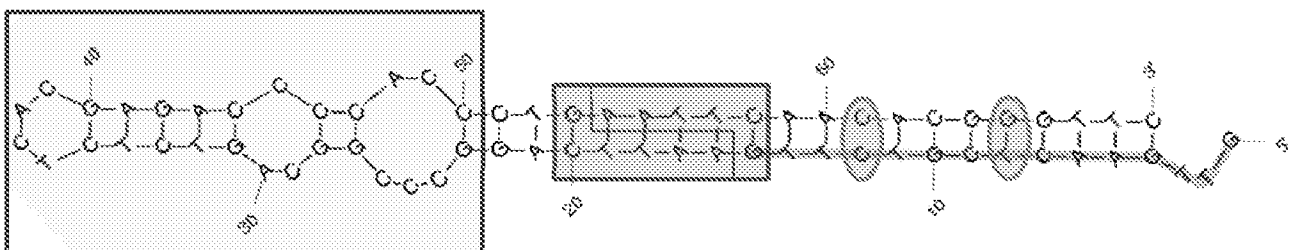


FIGURE 3

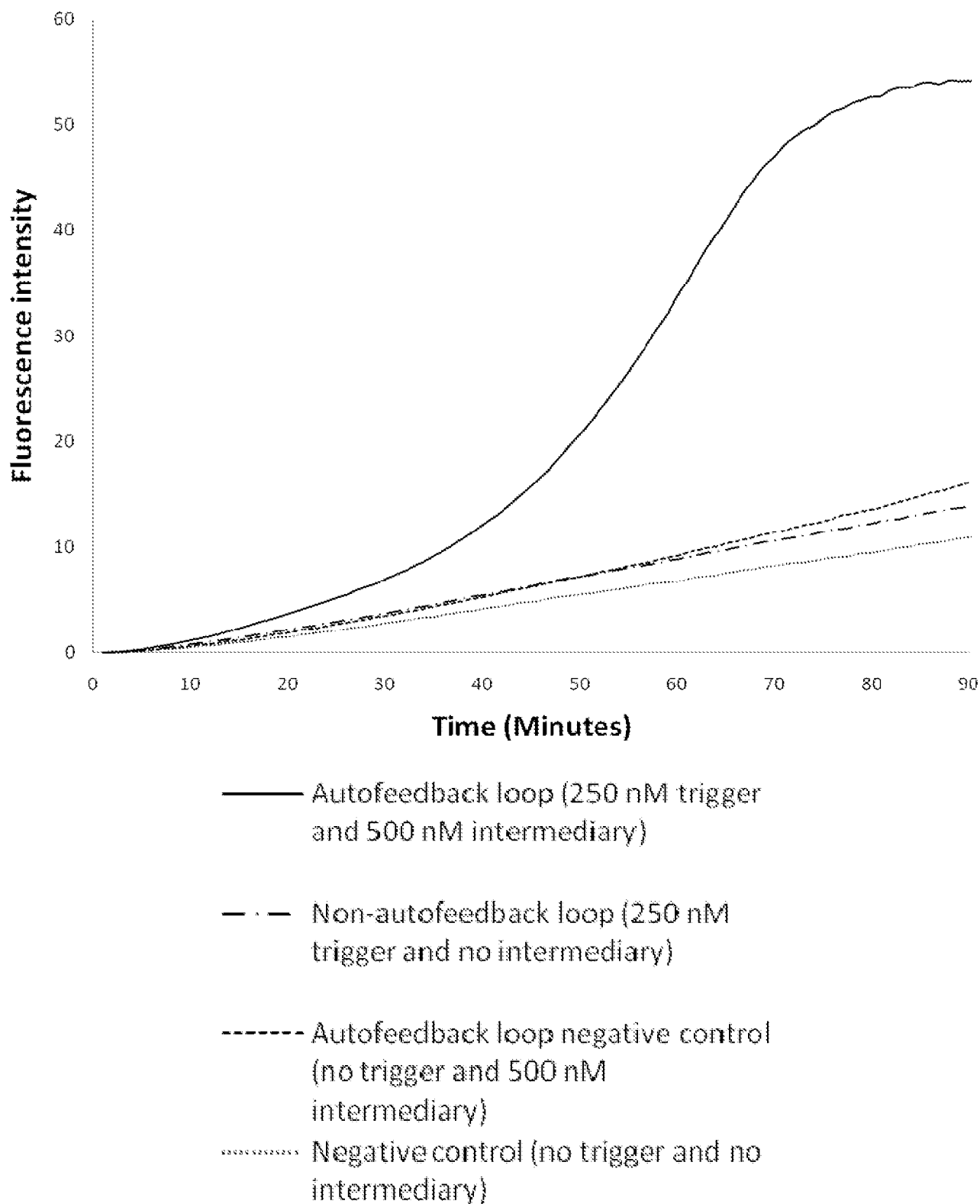


FIGURE 4

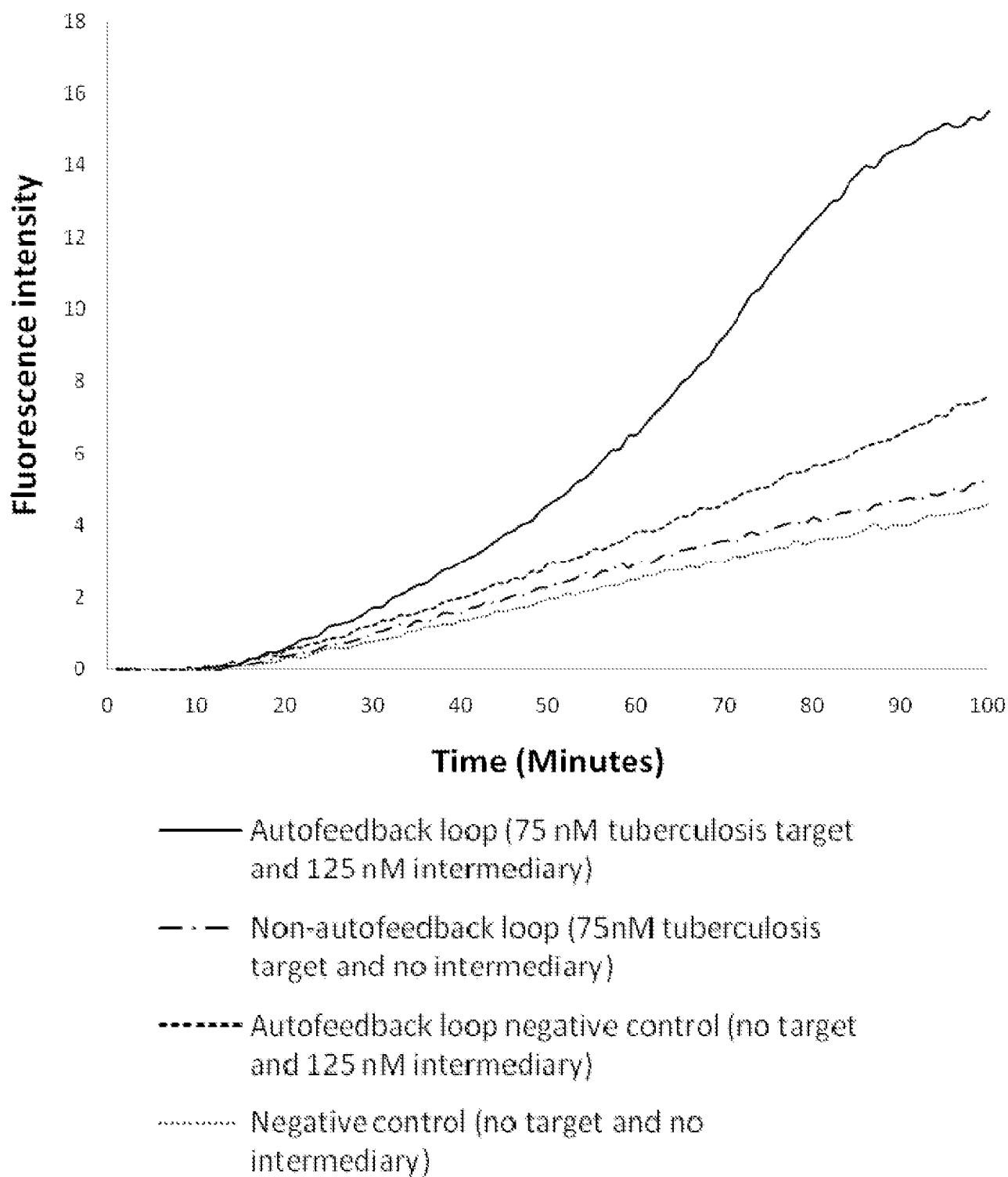


FIGURE 5

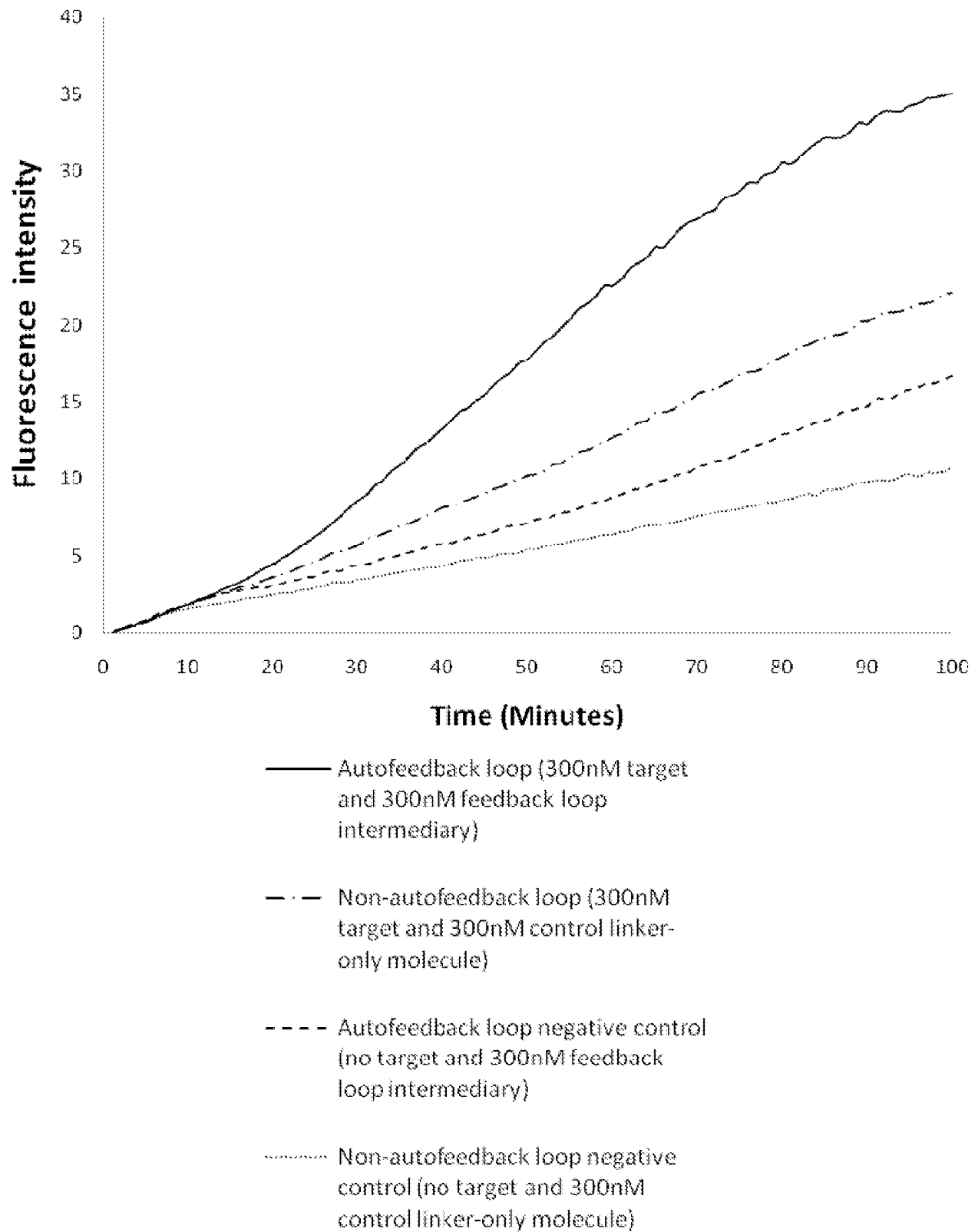


FIGURE 6

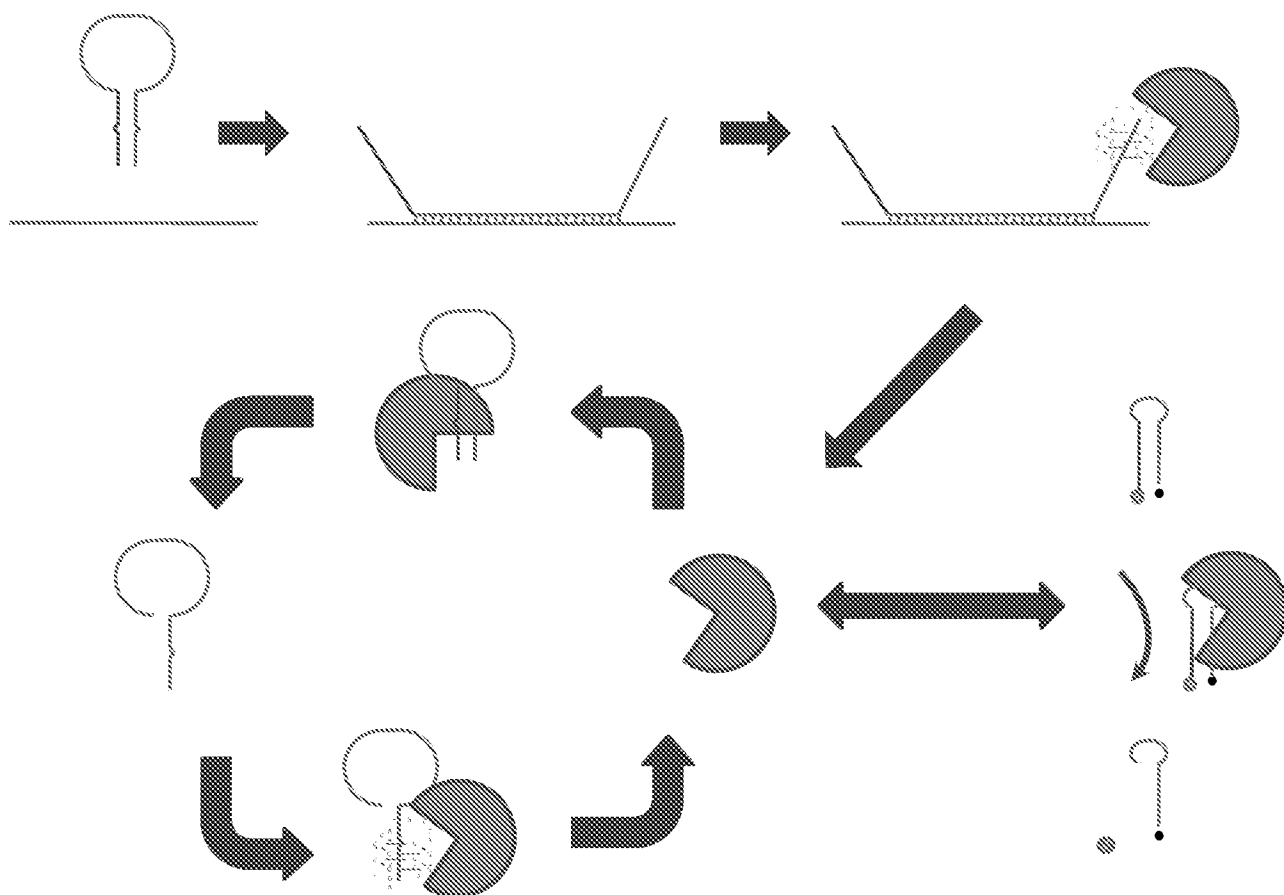


FIGURE 7

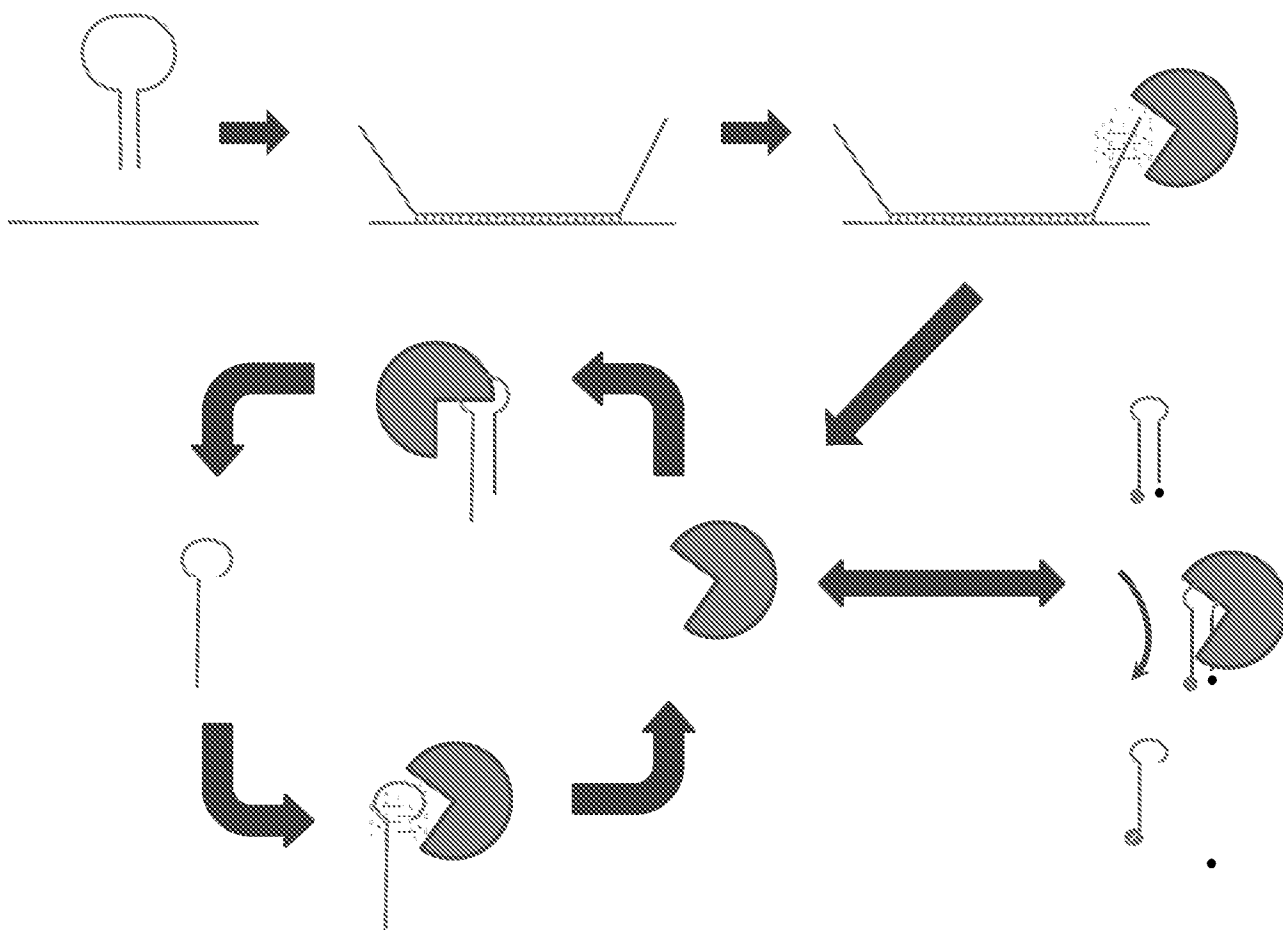


FIGURE 8

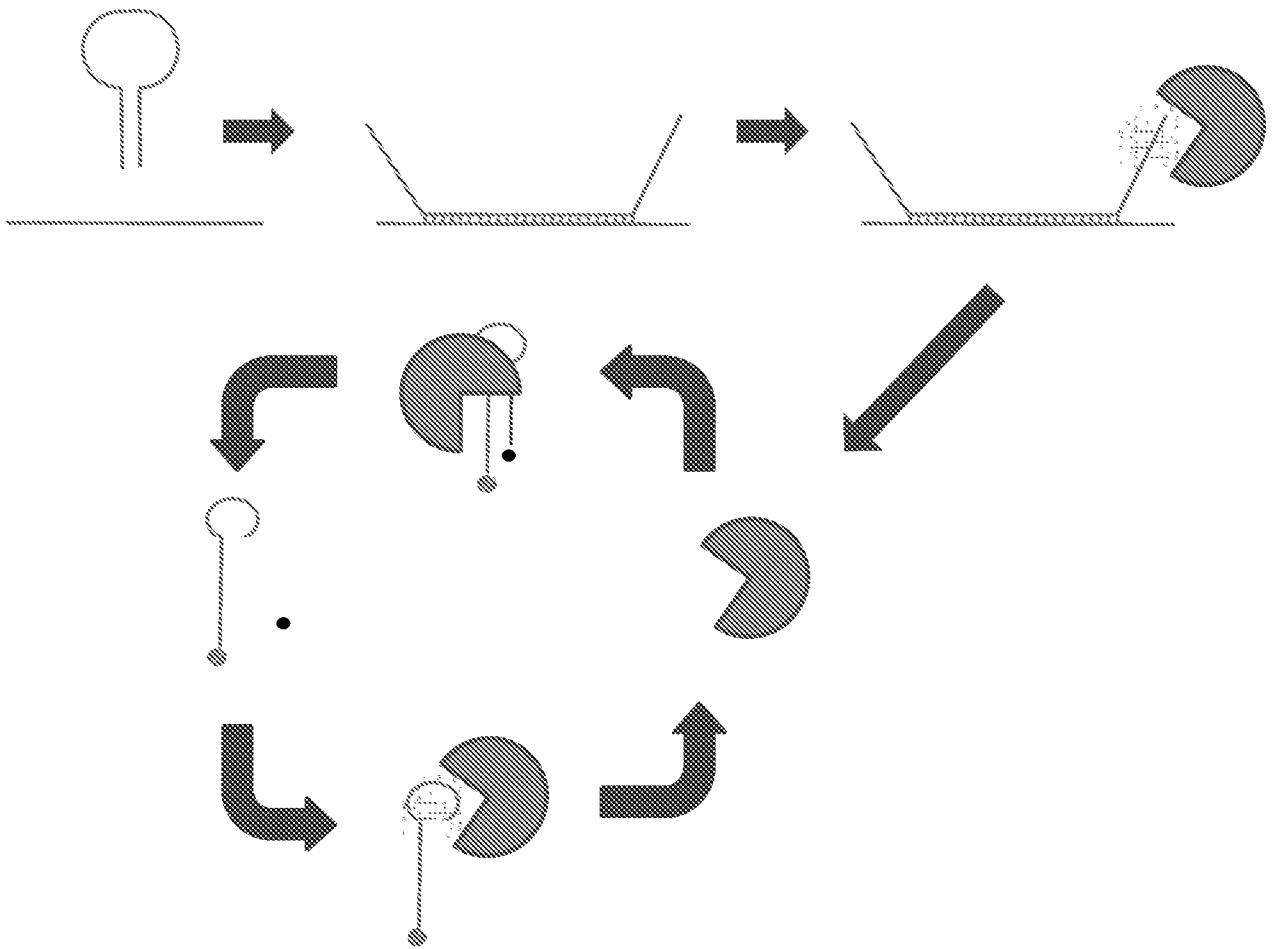


FIGURE 9

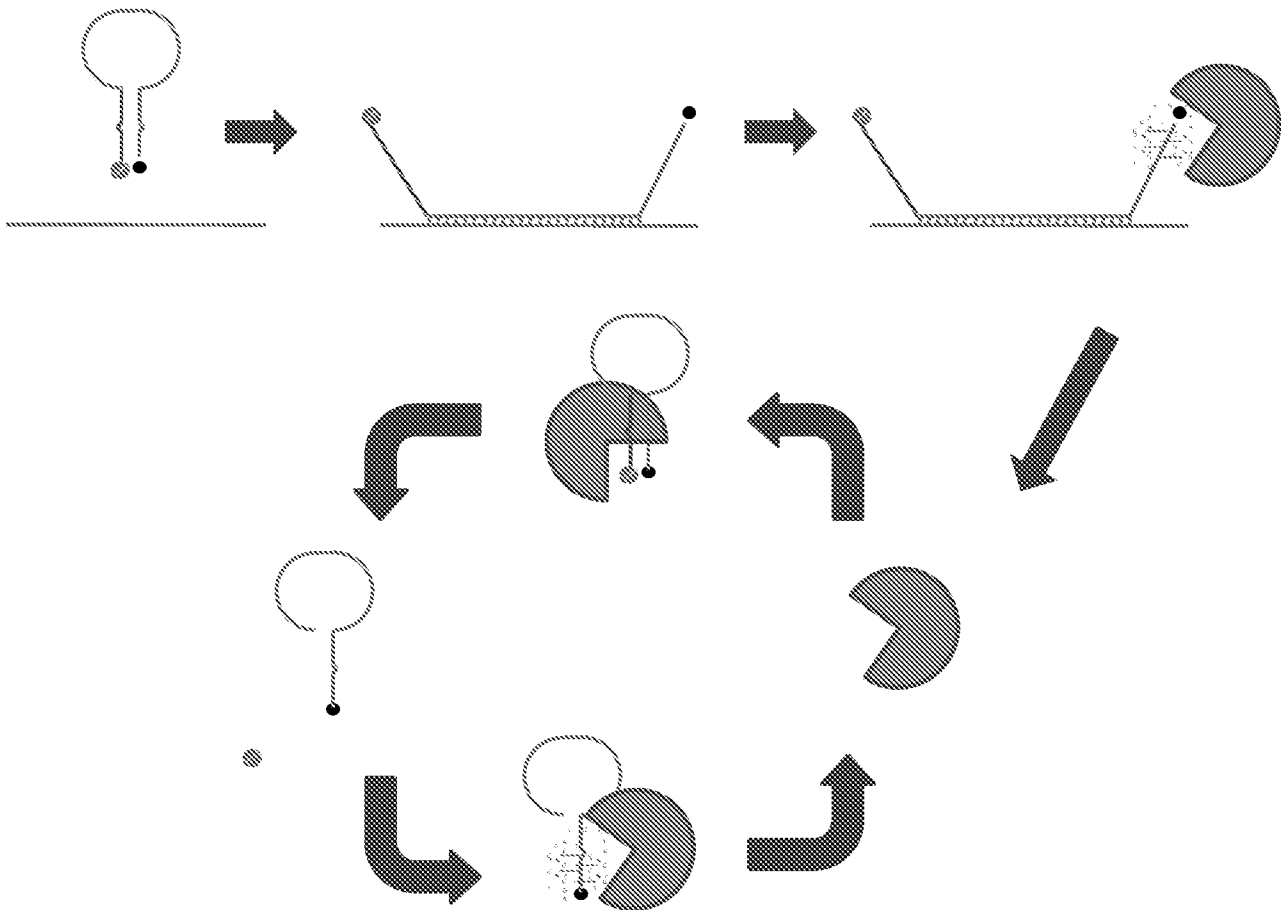


FIGURE 10

>Aptamer 3.8

CGAAGGGAA...TGCTAGGGGGGGTGGGTGTGA

>Aptamer 3.9

CACAGGGAGGGGTGCTA....GGGTGGGTATGT

>Aptamer 3.22

CGGGGTGGGTGTCCAGGGCGGGATGCTCG

>Aptamer 5.10

GCGGGGGGGGCATCGGCTCGGGGAGGGCTACATGAC

>Aptamer 5.2

GGGGGGGCATCGGCGATCATGAGCGGAGGAACCTAAATAC

>Aptamer 5.3

GGGACGTGGGGGGGCATCGGCTCCGCCTCATGG

>Aptamer 6.5

GGGGGGGCATCGTCGATTATGTAAGGGGCC

>Aptamer 6.6

GGGGGGGCATCGTCGGGGGTGCGT.GGCC

>Aptamer 6.13

GGGGGGGCATCGTCGAGACGGGGA..GGCCCT

FIGURE 11

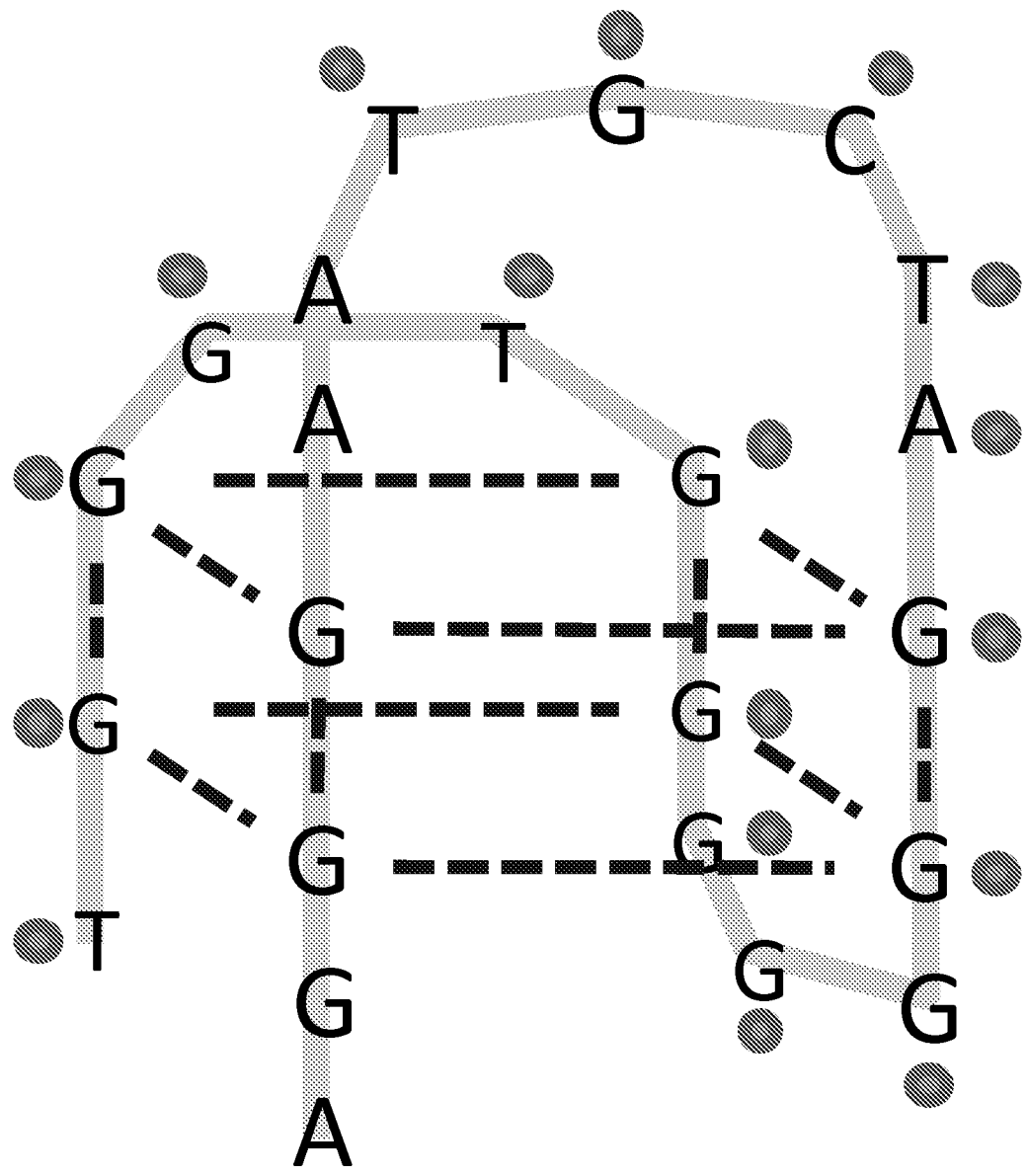


FIGURE 12

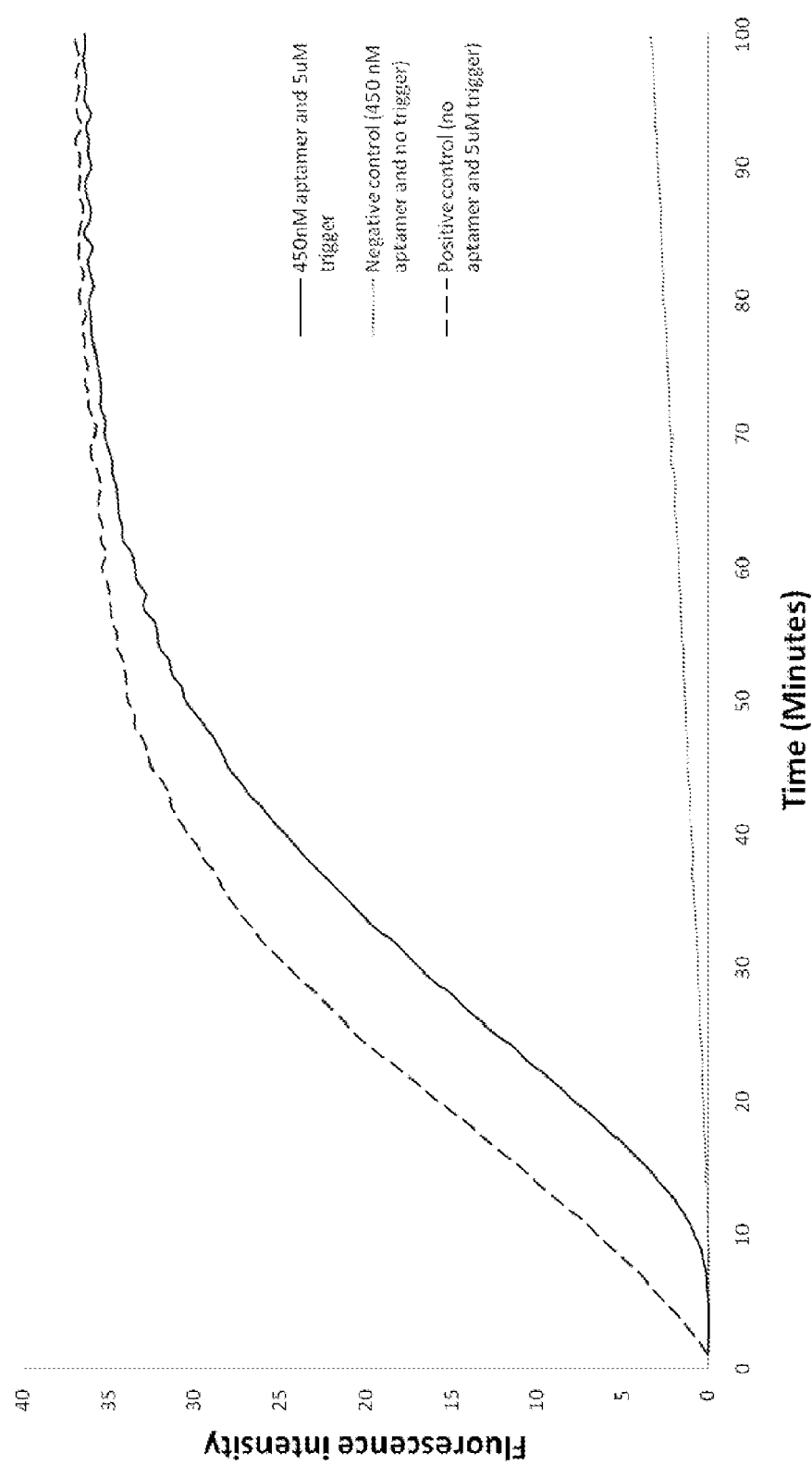


FIGURE 13

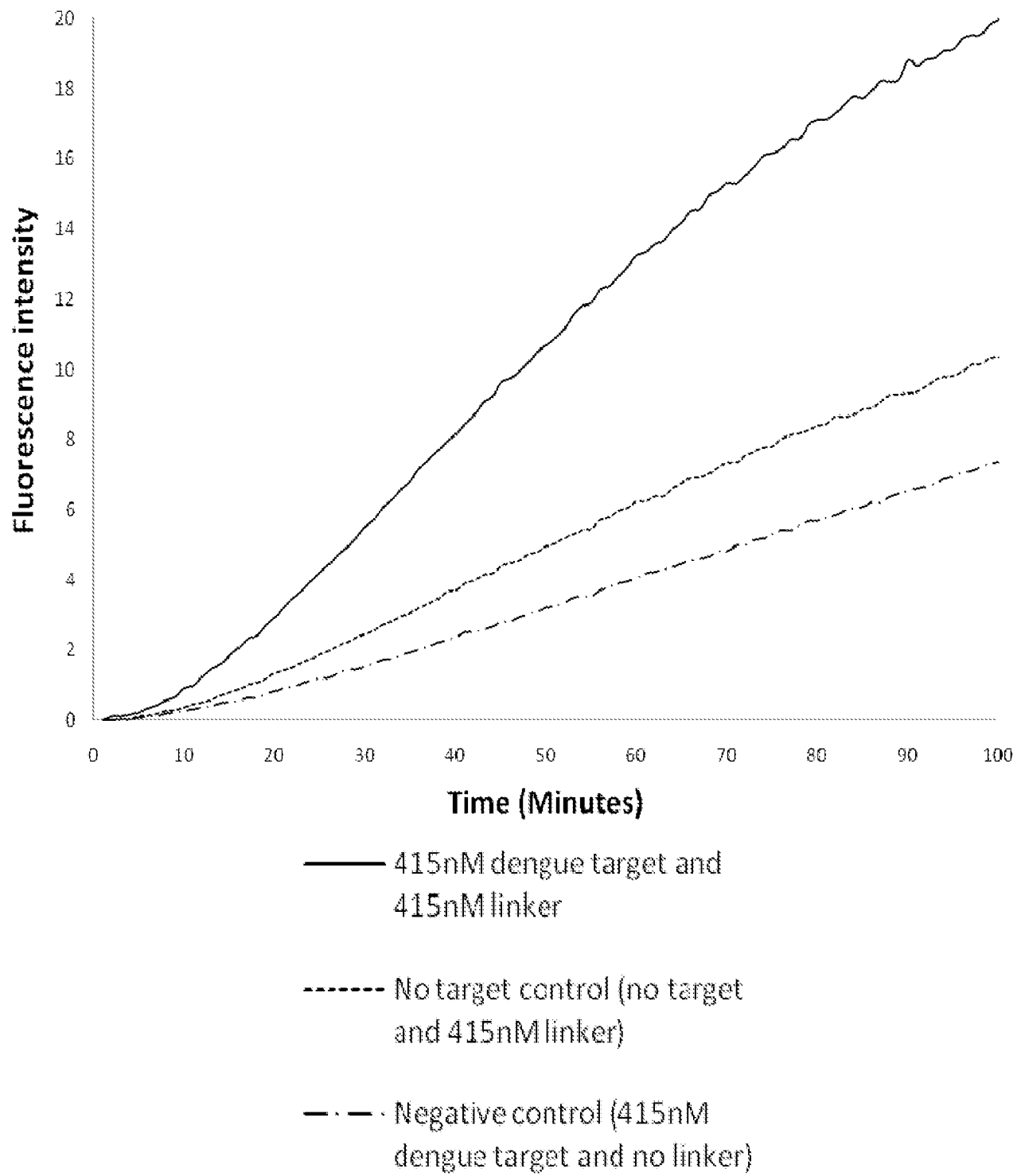


FIGURE 14

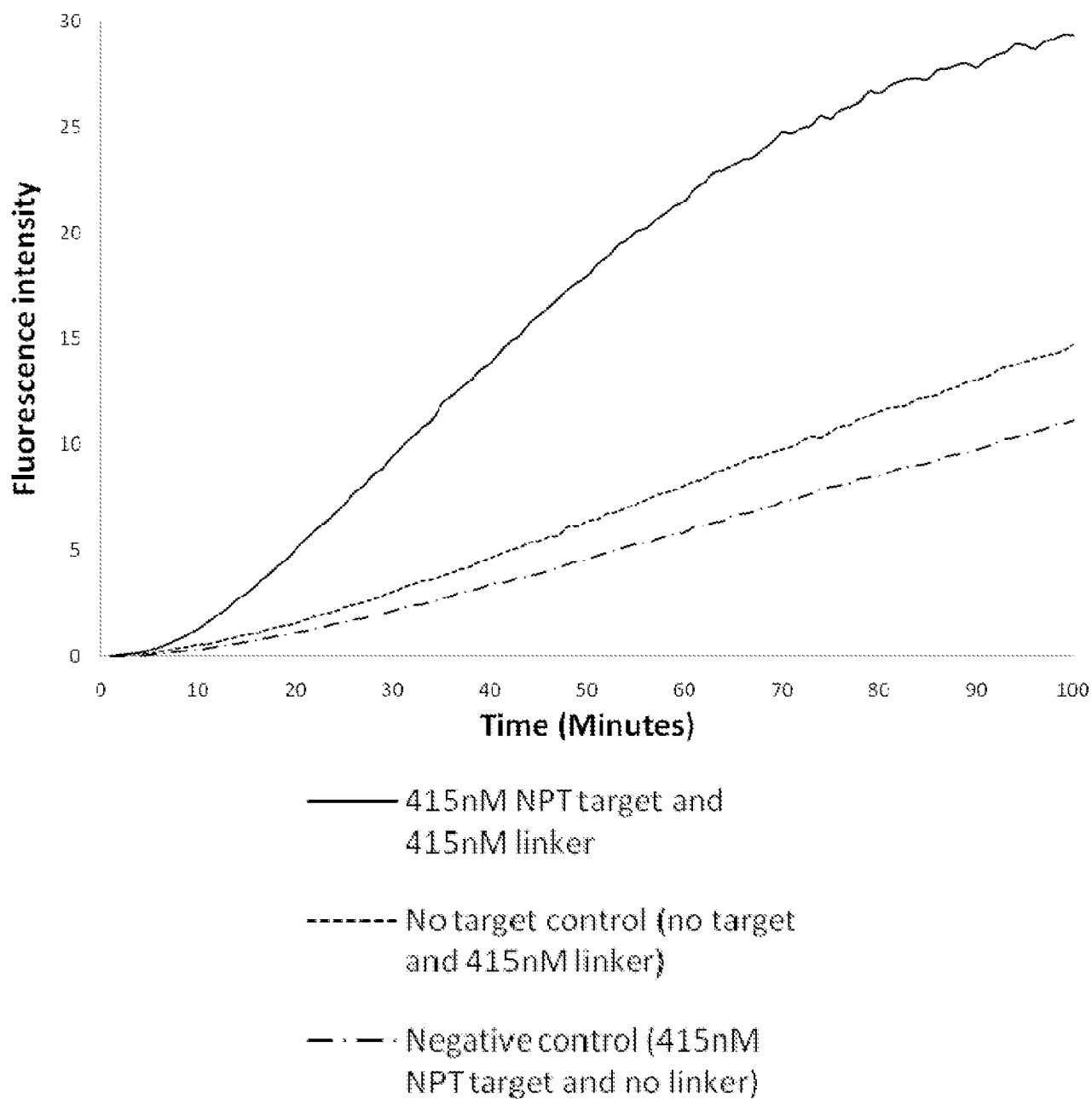


FIGURE 15

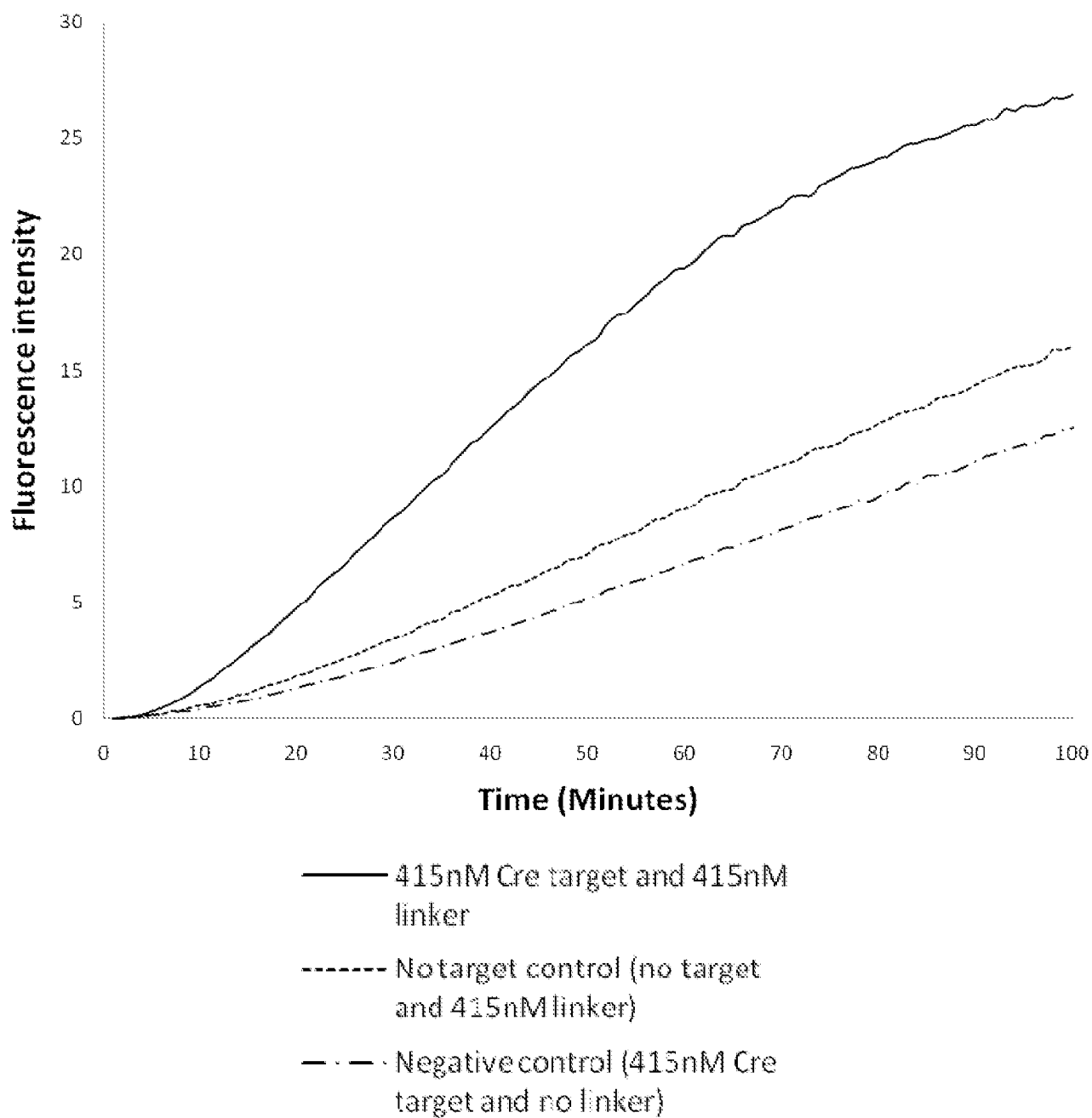


FIGURE 16

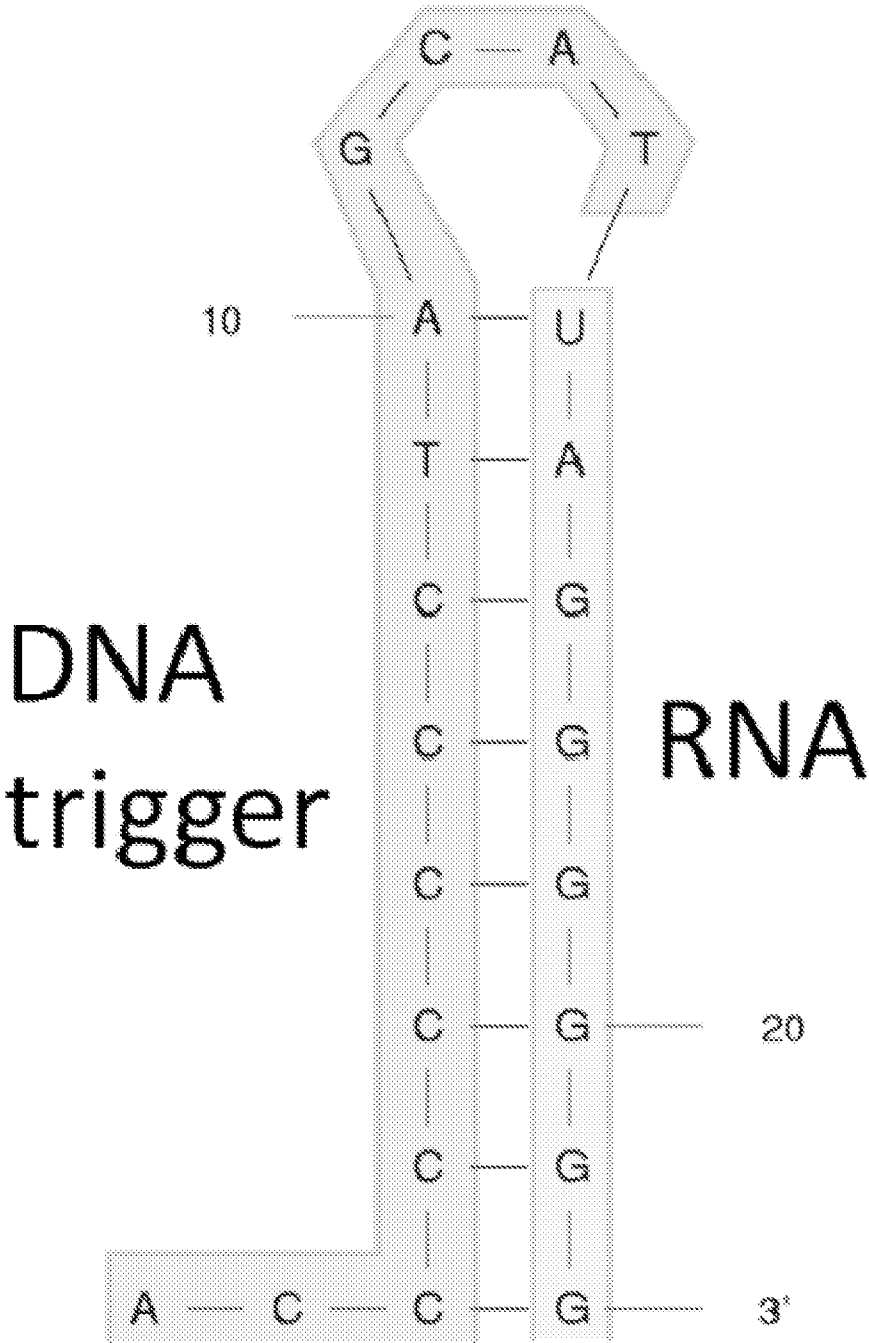
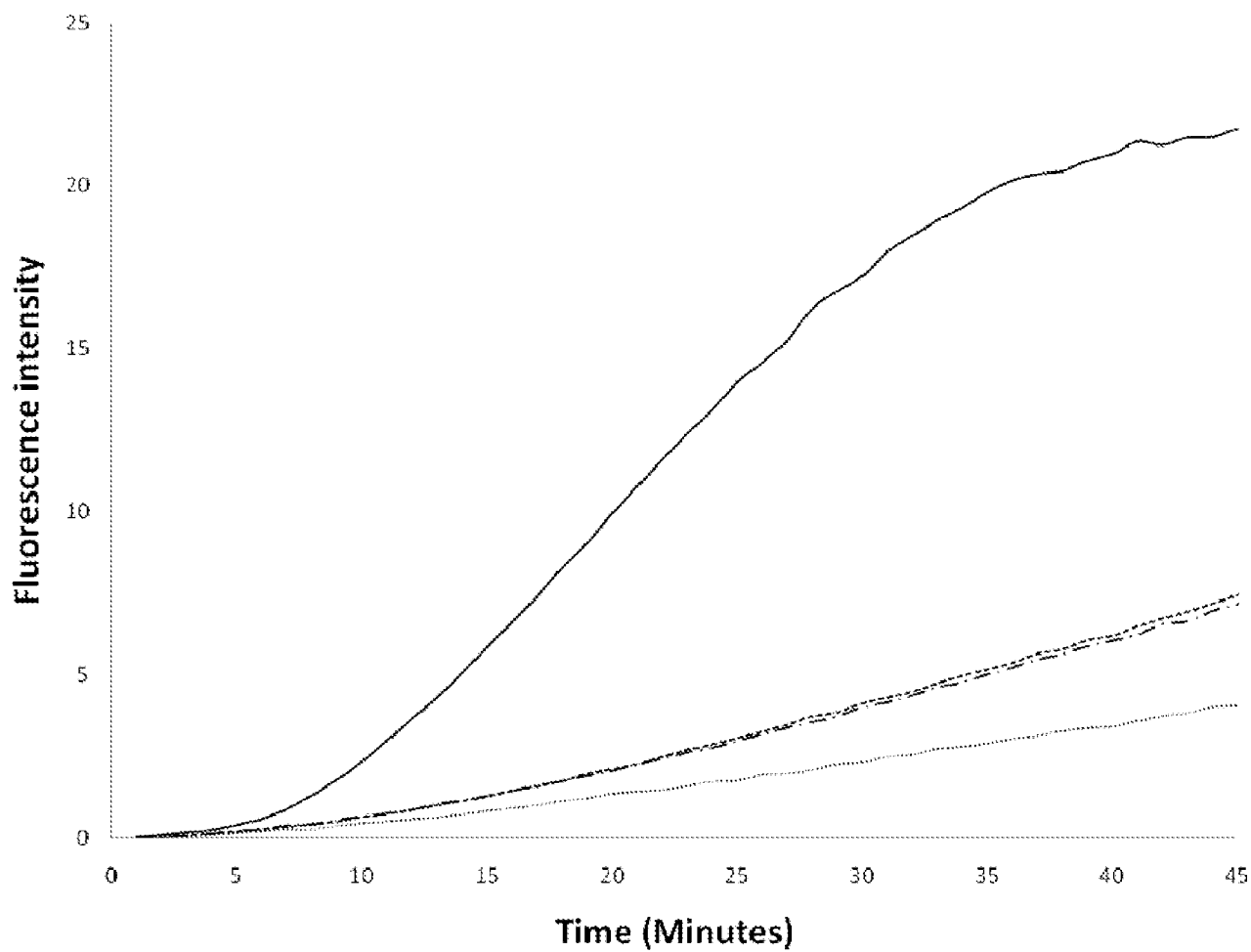


FIGURE 17



- Autofeedback loop (110nM trigger and 310nM intermediary)
- - Non-autofeedback loop (110nM trigger and no intermediary)
- Autofeedback loop negative control (no trigger and 310nM intermediary)
- Negative control (no trigger and no intermediary)

FIGURE 18

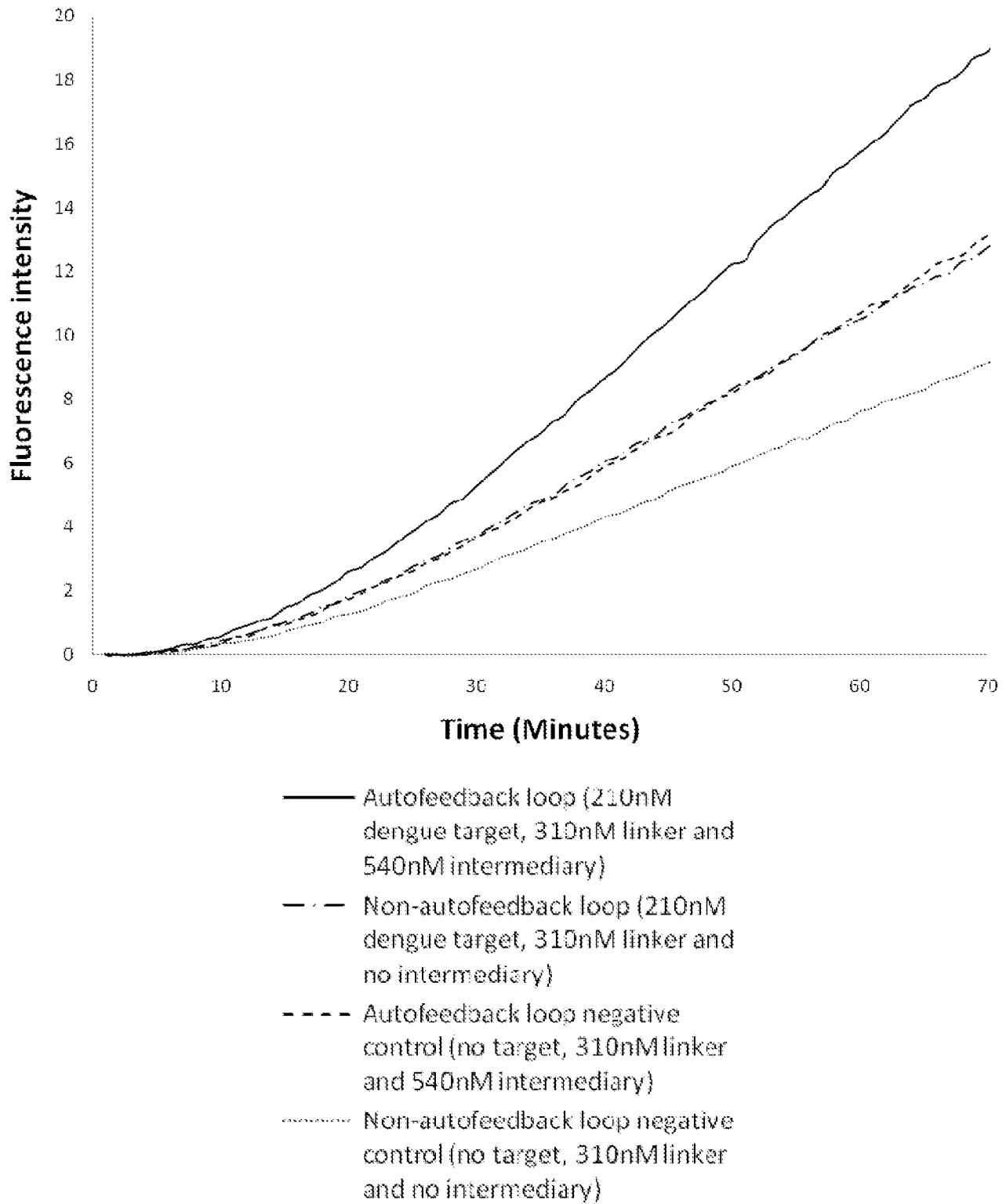
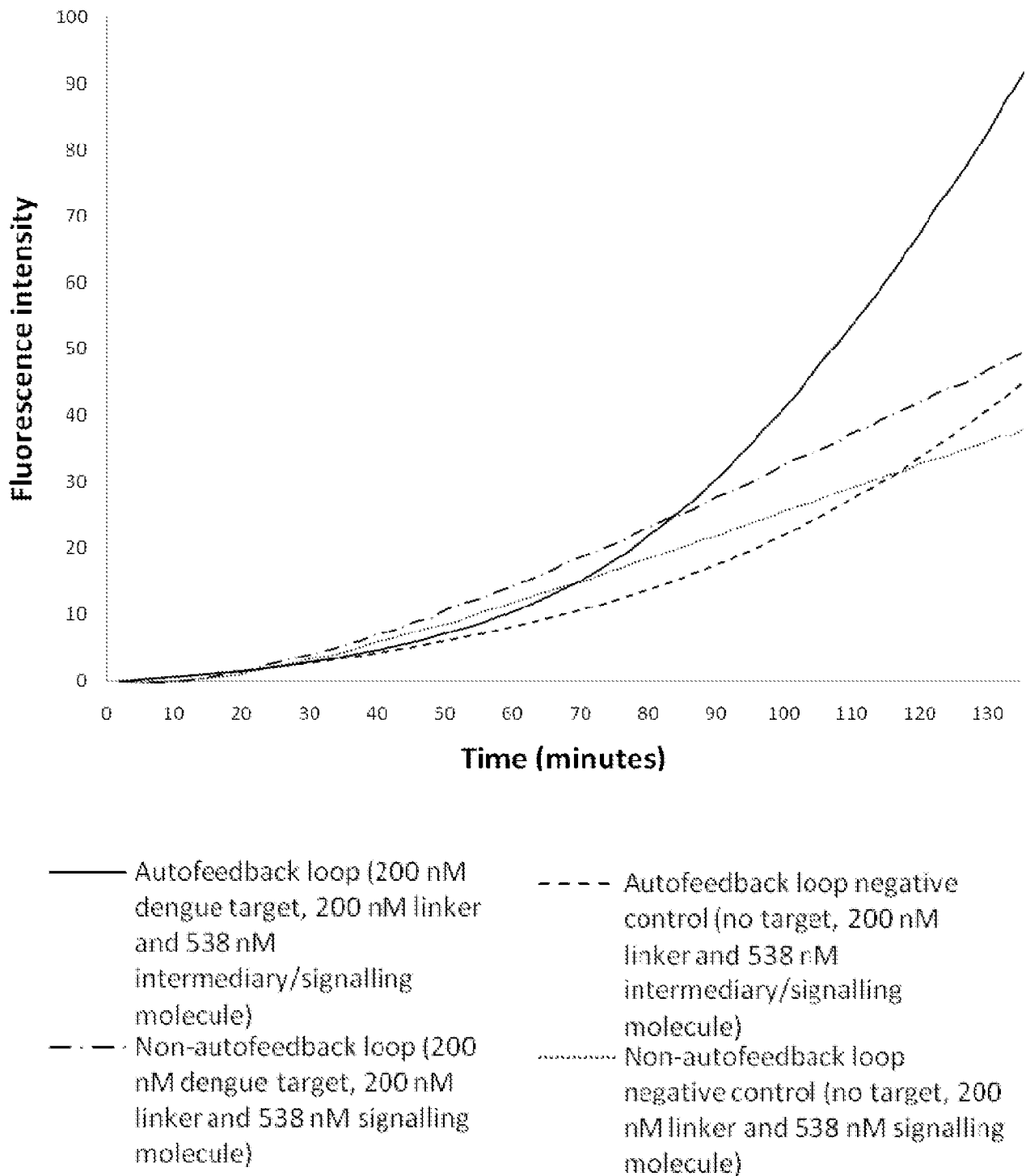


FIGURE 19



INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2009/000768

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12Q 1/68 (2006.01)

C12Q 1/48 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, HCA, BIOSIS, BIOTECHABS, EPODOC, WPI (aptamer, endonuclease, exonuclease, deoxyribonuclease, ribonuclease, nuclease, RNase H, RNase, DNase, CEL 1, EcoR1, inhibit, repress, impede, stop, reduce, decrease)
GENOMEQUEST (SEQ ID No's 1-3, 10-22 and 31)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/125094 A2 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 23 November 2006 See whole document especially Abstract; Pages 2 and 18; Claim 32	51, 57-59
X	SOMASUNDERAM A. et al, "Combinatorial Selection, Inhibition and Antiviral Activity of DNA Thioaptamers Targeting the RNase H Domain of HIV-1 Reverse Transcriptase", Biochemistry, 2 August 2005, Vol. 44, No. 30, pp 10388-10395 See whole document especially Abstract; Results; Figure 3	51, 57-59
X	THIVIYANATHAN V. et al, "Combinatorial selection and delivery of thioaptamers", Biochemical Society Transactions, 2007, Vol. 32, No. Pt 1, pp 50-52 See whole document especially Abstract	51, 57-59

☒ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"E" earlier application or patent but published on or after the international filing date

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"O" document referring to an oral disclosure, use, exhibition or other means

"&" document member of the same patent family

"P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search
21 July 2009

Date of mailing of the international search report
27 JUL 2009

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2009/000768

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PILEUR F. et al, "Selective inhibitory DNA aptamers of the human RNase H1", Nucleic Acids Research, 2003, Vol. 31, No. 19, pp 5776-5788 See whole document especially Abstract; Page 5782	51, 57-59
A	WO 1996/040159 A1 (MERCK & CO., INC.) 19 December 1996 See whole document especially Page 5; Claim 20	
A	WO 2008/122088 A1 (LIFEPRINT AUSTRALIA PTY LTD) 16 October 2008 See whole document especially Abstract	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2009/000768

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	2006/125094	US	2006281702		
WO	1996/040159	CA	2223549	EP	0831846
		US	6111095	US	5861501
				US	6369208
WO	2008/122088	NONE			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.					
END OF ANNEX					