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(56) Documents Cited
WO 1992/001813 A **US 5854033 A**
Biotechniques; Vol 30 (3), pp 584-588 (2001). Myer & Day
Nucl Acid Res; Vol 28 (12), pp E58 (2000). Antson et al.
Nucl Acid Res; Vol 26, pp 5073-5078 (1998). Banér et al.

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Other: **ONLINE: WPI, EPODOC, JAPIO, MEDLINE, EMBASE, BIOSIS, SCISEARCH, CAPLUS**

(54) Abstract Title
Nucleic acid amplification method

(57) A method of amplifying a nucleic acid product comprising the steps of providing a first generation amplification product which comprises a concatamer of the sequence to be amplified, monomerising the amplification product, and carrying out a further amplification of the thus-formed amplification product to form a second generation amplification product. In a preferred embodiment, the monomers are ligated to form circles prior to further amplification. Preferably, the first generation amplification product is a linear rolling circle amplification (RCA) product, and the further amplification is RCA. A further embodiment provides a method of nucleic acid amplification employing probes to indicate the extent of amplification, and yet another embodiment defines a method of removing non-circularised probes during amplification.

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Fig. 1

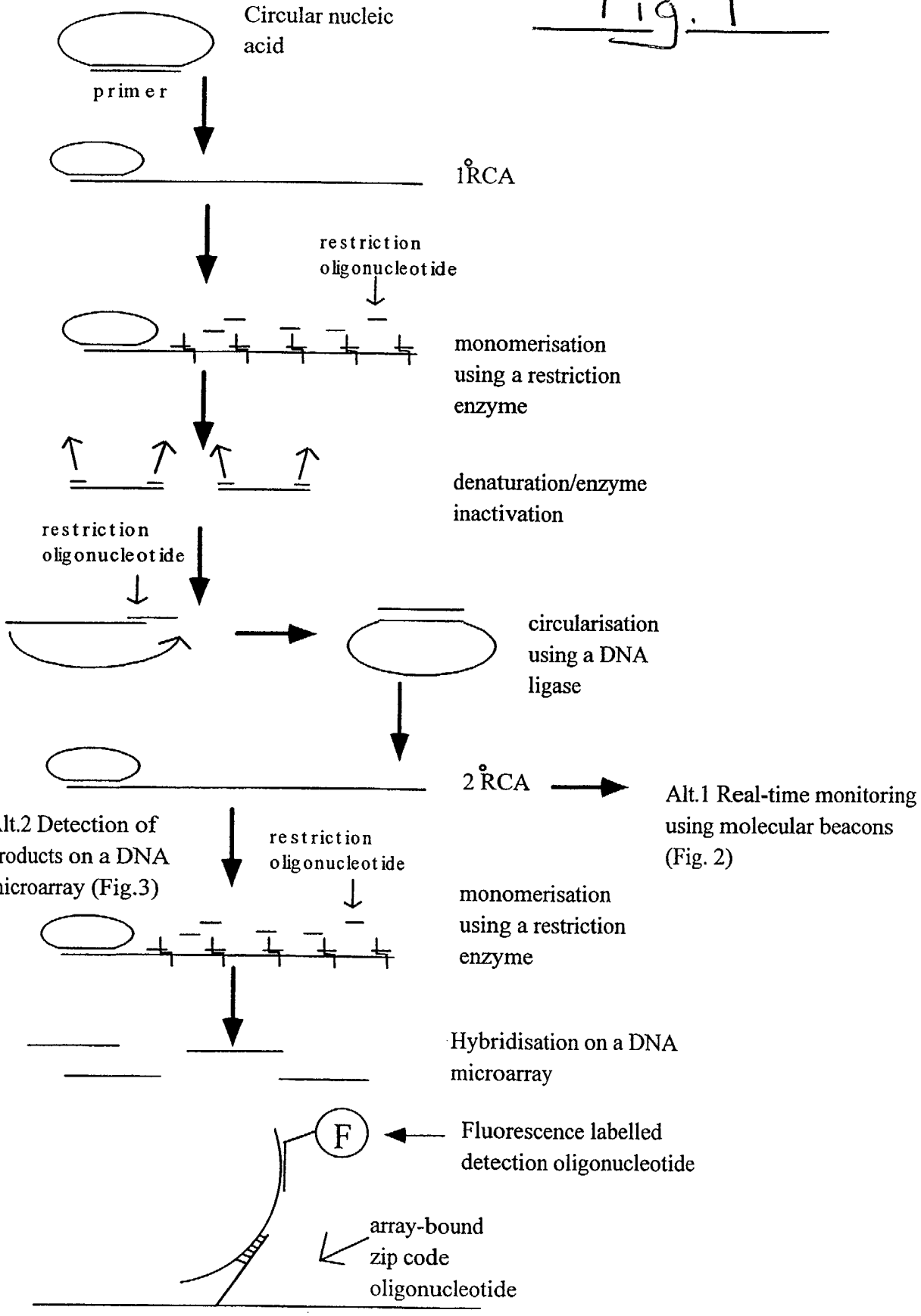
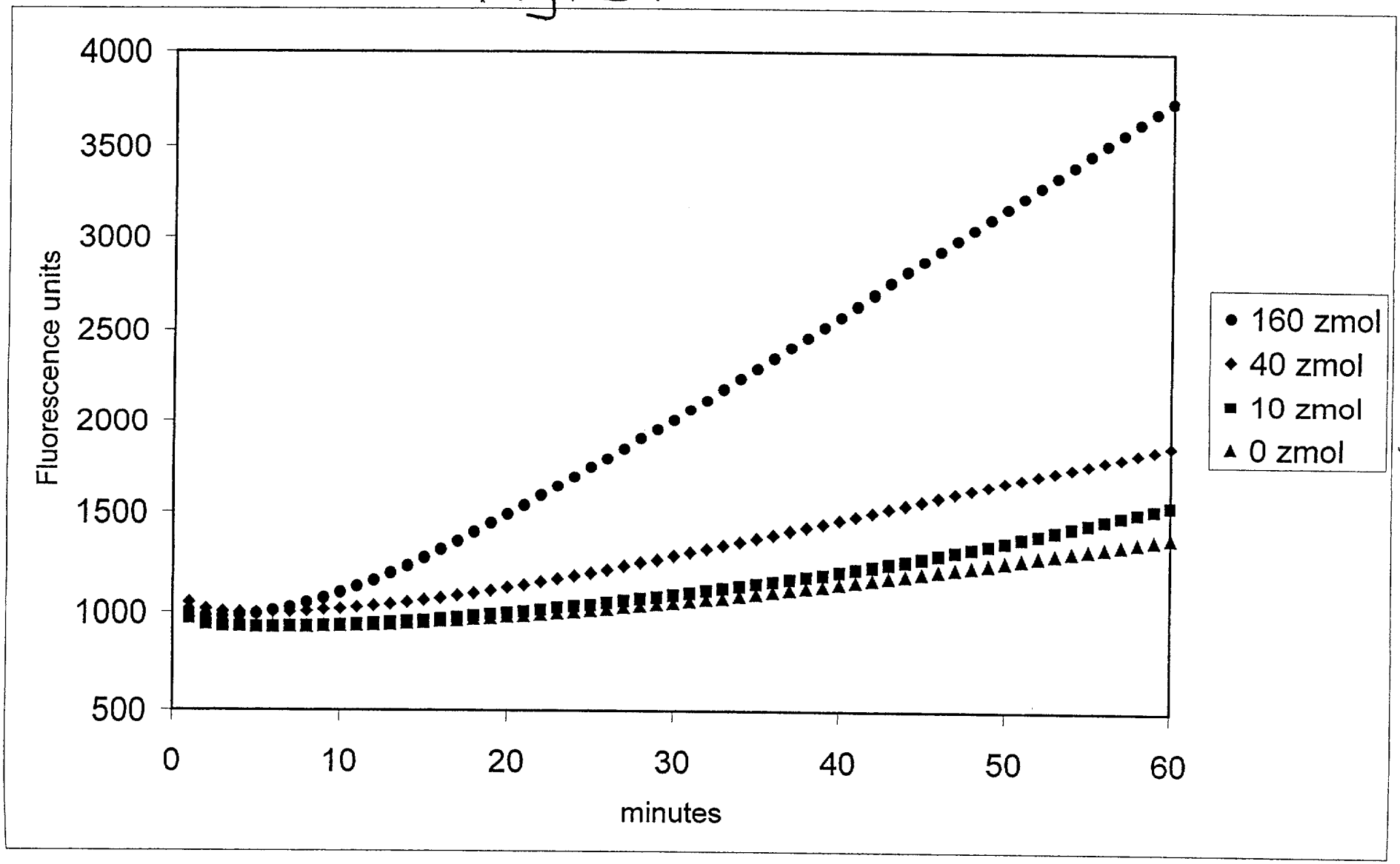

Circular nucleic acid
primer
1°RCA
restriction oligonucleotide
monomerisation using a restriction enzyme
denaturation/enzyme inactivation
restriction oligonucleotide
circularisation using a DNA ligase
2°RCA
Alt.1 Real-time monitoring using molecular beacons (Fig. 2)
Alt.2 Detection of products on a DNA microarray (Fig.3)
restriction oligonucleotide
monomerisation using a restriction enzyme
Hybridisation on a DNA microarray
F
Fluorescence labelled detection oligonucleotide
array-bound zip code oligonucleotide

Fig. 2A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3500
3000
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minutes
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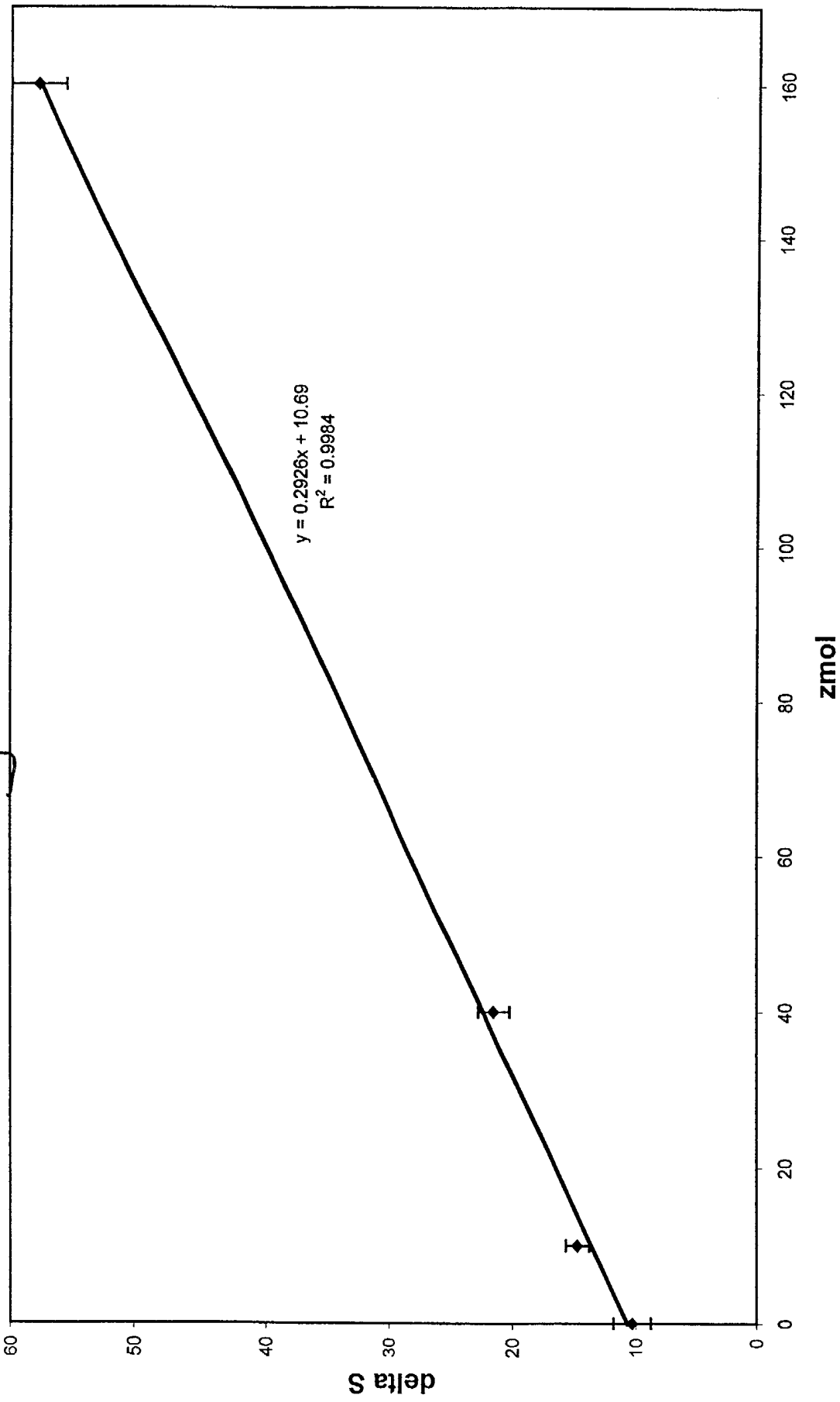
10 zmol
0 zmol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R² = 0.9984
delta S
zmol
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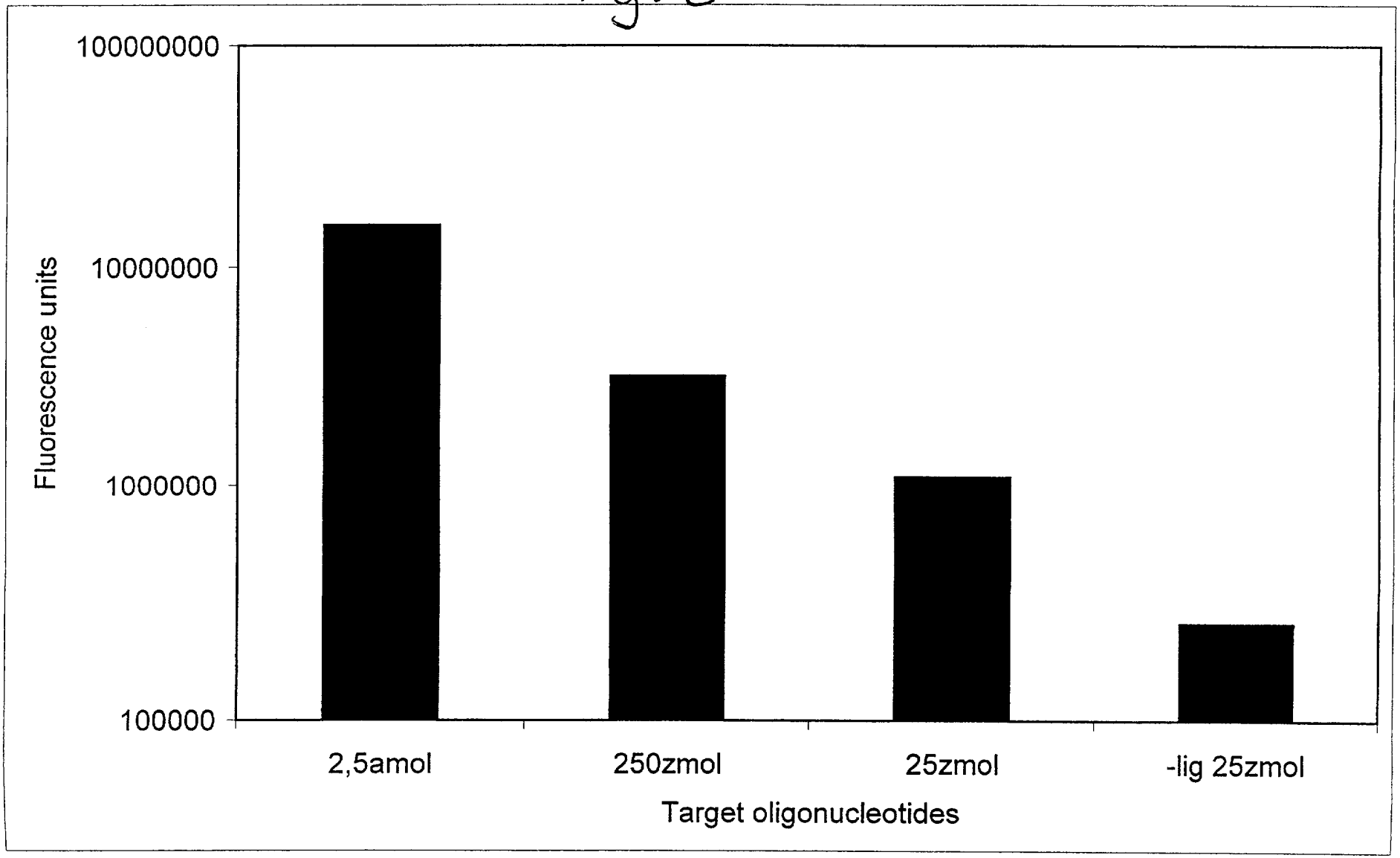
40
50
60
0
20
40
60
80
100
120
140
160

Fig. 2B

Fig. 3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10000000
1000000
100000
Fluorescence units
2,5amol
250zmol
25zmol
-lig 25zmol
Target oligonucleotides

Fig 4

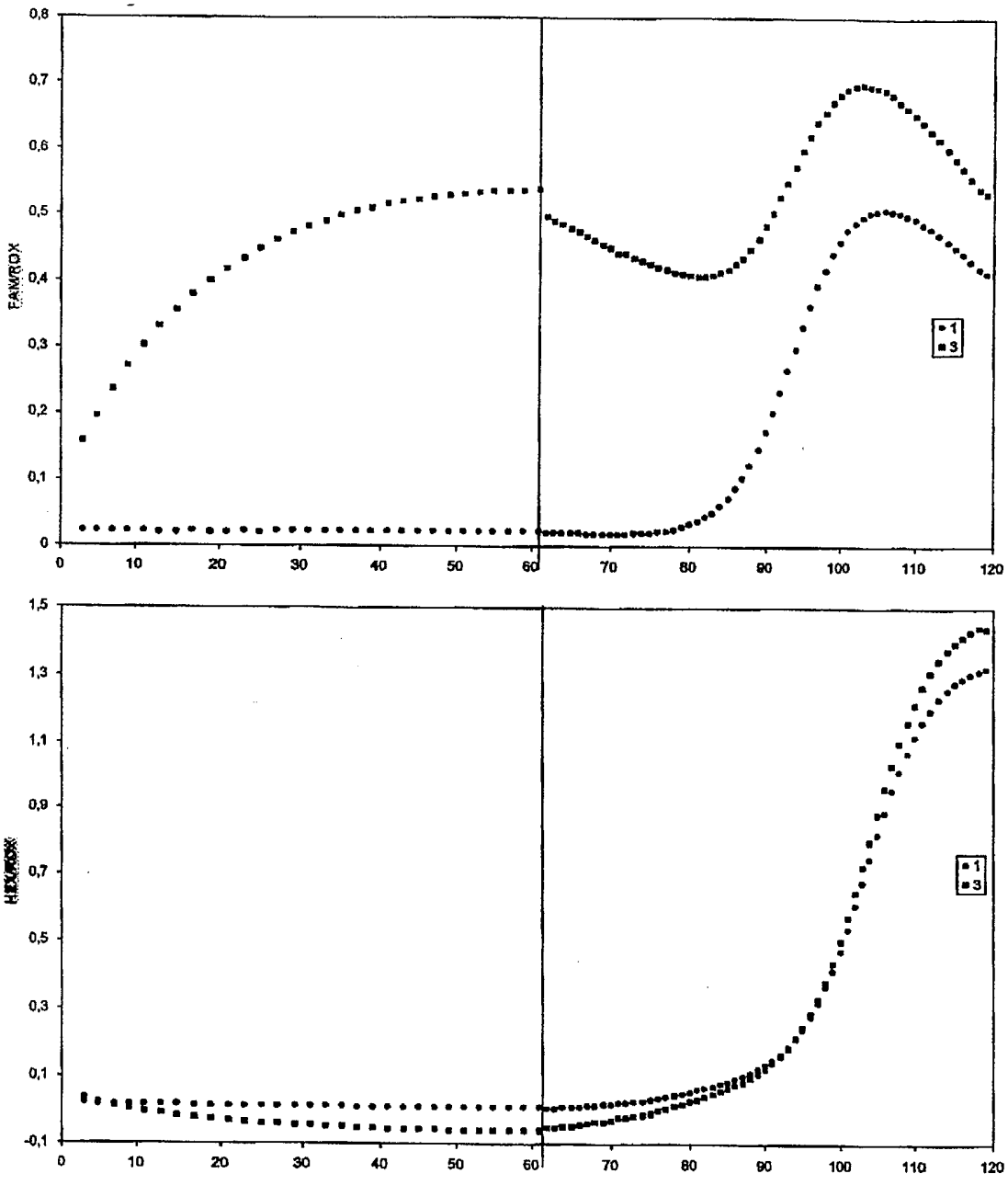


Fig. 5

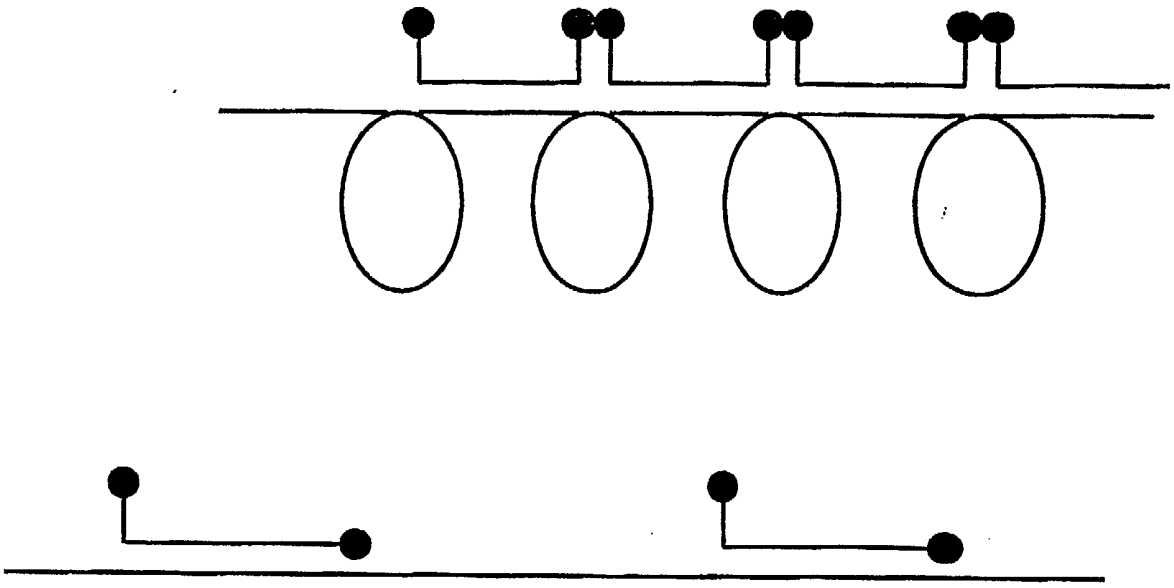
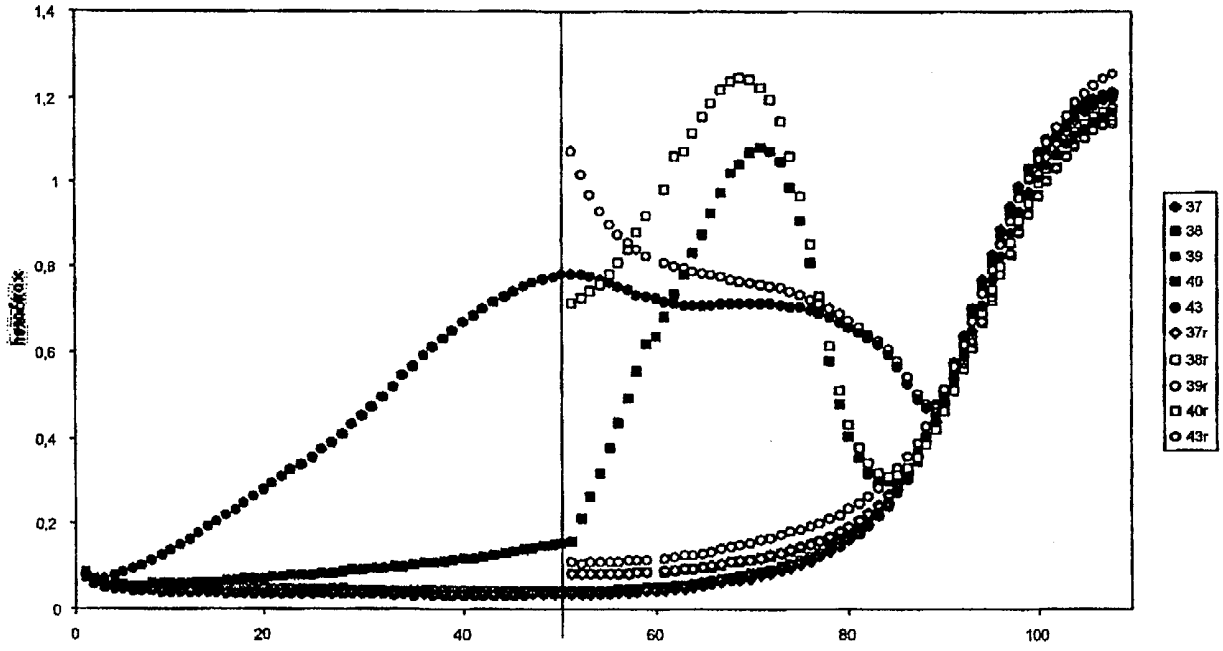


Fig. 6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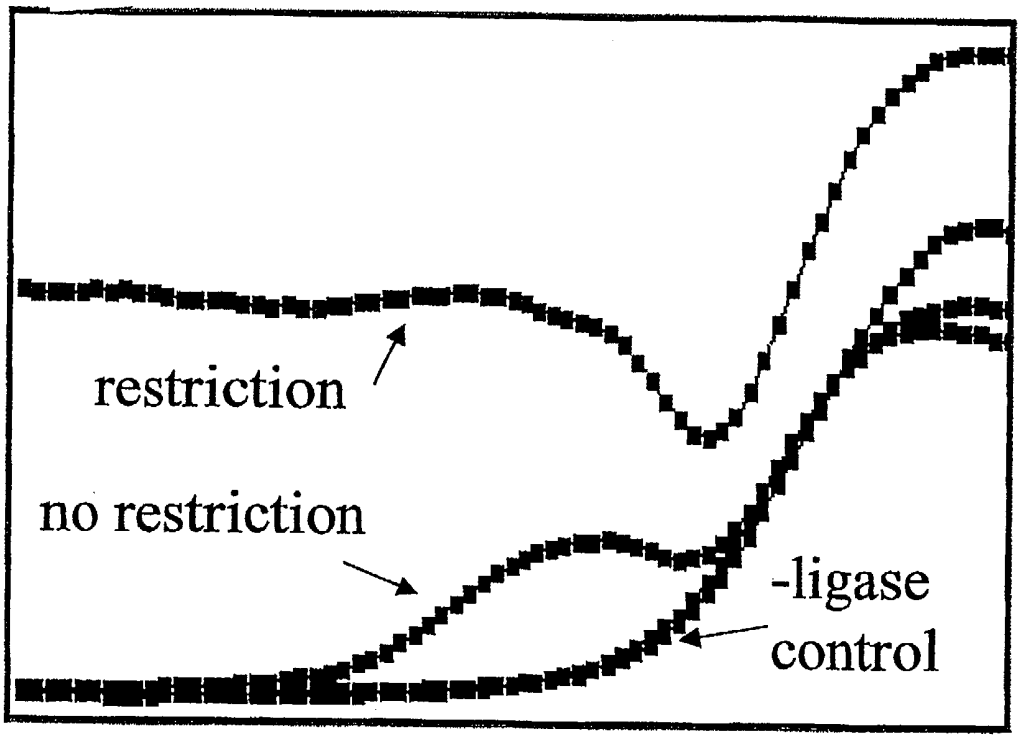
37r
38r
39r
40r
43r

Fig. 7



Real-time monitoring of rolling circle replication reactions using a modified molecular beacon design

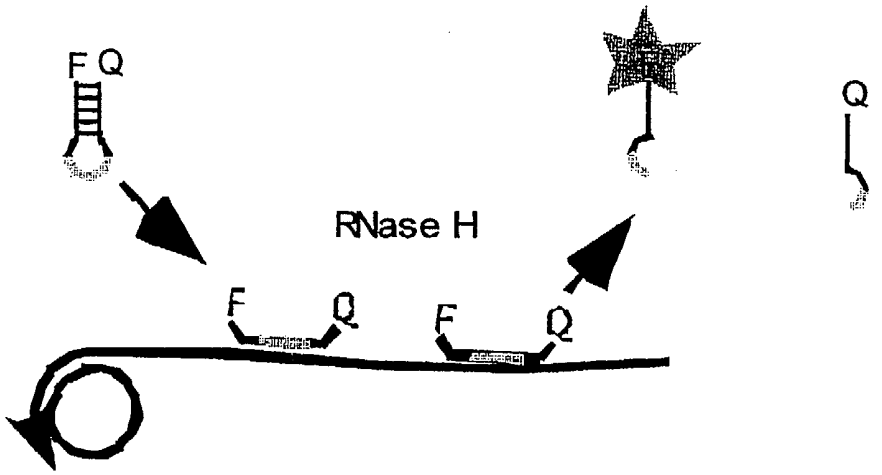


Fig. 8

NUCLEIC ACID AMPLIFICATION METHOD

Introduction

This invention relates to the amplification, detection and quantification of nucleic acids. More particularly, the present invention relates to the amplification, detection and quantification of circular nucleic acids.

Over the last 20 years nucleic acid amplification has become a common technique in the identification of genetic disorders, genetic variants or for the identification of infectious agents.

Many methods exist for the amplification of nucleic acids. These include the polymerase chain reaction (PCR), the ligase chain reaction (LCR), both of which require thermal cycling, the transcription based amplification system (TAS), the nucleic acid sequence based amplification (NASBA), the strand displacement amplification (SDA), the invader assay, rolling circle amplification (RCA), and hyper-branched RCA (HRCA).

In the description that follows, the present invention will be described with reference to its preferred use in rolling circle amplification method (Lizardi *et al.*, 1998), US5854033, US6124120, US6143495, US6183960, US6210884. However, it is not intended to be limited thereto since the invention may find equal utility in other nucleic acid amplification methods, especially other circular amplification methods. The circular nucleic acids referred to in the invention can be circularised probes, circularised target nucleic acids, circular reporter nucleic acids, plasmids, or circular target nucleic acids. The present invention can be used for nucleic acid sequence analyses for e.g. genotyping, finding mutations, identification of infectious agents, molecular diagnostics, genetic analyses and forensics.

RCA results in a linear amplification of circularised probes and is therefore of limited sensitivity. Typically an RCA produces about 1000 copies of each circularised probe molecule per hour.

It is an object of the present invention to improve the sensitivity of nucleic acid amplification processes, especially RCA processes.

Statements of Invention

One means of improving the sensitivity of an amplification process, such as for example a RCA process, is to increase the signal obtained during the process. This may be done by turning the first generation RCA product into a new generation of monomers which themselves are amplified in a further amplification reaction. Accordingly, in a first aspect, the invention relates to a method of amplifying a nucleic acid product, comprising the steps of:

- providing a first generation amplification product, typically by RCA, which product comprises a concatamer of a sequence to be amplified;

- monomerising the amplification product; and

- carrying out a further amplification of the thus-formed monomers to form a second generation amplification product.

Preferably, the monomers are ligated to form circles prior to further amplification of the circles, in which case the further amplification is ideally RCA. The sequence to be amplified may be a probe sequence, or it may comprise cDNA, genomic DNA or RNA sequences.

In one embodiment of the invention, monomerisation of the first generation amplification product is achieved using a restriction enzyme and an oligonucleotide complementary to the first generation amplification product, wherein the restriction enzyme cleaves any first generation amplification product/oligonucleotide hybrids. Typically, oligonucleotide is added in excess to a number of monomers contained in the first generation reaction product.

Alternatively, the sequence to be amplified may include a catalytically active sequence which enables monomeristaion of the first generation amplification product.

Typically, the first generation amplification product is produced in a first generation amplification step, which step utilises a polymerase enzyme,

wherein the method includes an initial step of inactivating the polymerase enzyme. Ideally the method is carried out in isothermal conditions.

When monomerisation of the first generation amplification product is achieved using oligonucleotides and restriction enzymes, oligonucleotide is added in excess to the number of monomers present in the RCA reaction product. Most of the oligonucleotides that will hybridise to the monomers after a denaturation (and enzyme inactivation) step will be non-cleaved. Hence, the monomers will become circularised upon hybridisation to non-cleaved oligonucleotides and ligase treatment, because circularization is favoured (at any practical concentrations) over di- or multi-merisation due to the intramolecular nature of this hybridization reaction.

This new set of circles are now able to serve as templates for subsequent rounds of RCA, primed e.g. by the same oligonucleotide serving as template for ligation. Therefore, instead of a linear amplification, this procedure will generate a (X_1 x X_2 .....x X_n) amplification, where "X" is the number of monomers synthesized in each RCA, and "n" is the number of rounds of circularisations, RCAs, and digestions. The procedure should be of particular value for applications where a large set of different circular nucleic acids is amplified by RCA, since the procedure should preserve the complexity of the initial set of circular nucleic acids. This is due to the intramolecular nature of the circularization reactions, and because the replication of the circularised nucleic acids is contiguous and processive (one circle-> one product carrying thousands of copies of the circle). Furthermore, the reaction should not be product inhibited or primer limited, and does not recruit more polymerases during the reaction. Hence, the procedure should be independent of the number of different amplified circles. The product of the reaction is single stranded and readily available for detection via hybridisation probes. The linear nature of RCA reactions should ensure quantitative determination of target molecules.

The monomerisation can be obtained using a general sequence that is present in all of the different probes present in the reaction. In the second generation of the RCA, the risk that the product will become double-stranded

(see below) is minimal because all monomers (equivalent to linear probes in the first-generation RCA) will become circularised, due to the addition of excess cleavage/circularization oligonucleotides. Therefore, there should not be any sequences present in the second-generation RCA, complementary to, and able to prime synthesis on the second-generation RCA product.

The method of replicating a first, second, and maybe more generations of circularised sequences is applicable both for probe and target nucleic acid sequences. The product can be analysed by sequencing or by detection via using conventional techniques such as any of the methods described below.

In one preferred format, circularised amplification products are hybridised to different primers attached to a solid support, specific for each of the different products present in the reaction. The different primers are preferably designed to be as dissimilar as possible, i.e. they represent so-called zip-code- or tag-sequences, and they may be arranged in an array. The primers are used to initiate a localised RCA. By hybridising the circularised amplification products instead of the initial circular set of nucleic acids, the hybridisation kinetics and sensitivity are improved by several orders of magnitude. Moreover, if a localised RCA is monitored in real-time, a signal could be obtained with a very broad dynamic range, particularly suitable for expression analyses. If the complexity of the sample is very high with respect to differences in copy number and/or the number of different sequences, cross hybridisation between different tag-sequences will be difficult to avoid completely. Therefore, it would be valuable to increase the specificity of zip code recognition by using the solid support attached tag-sequence primers as template for circularisation. If the number of different nucleic acids is very high, a monomerisation procedure must be devised that allows for specific cleavage in each of many different tag-sequences. For this purpose, every sequence in the complex mix of circularisable nucleic acids may be equipped with a type IIs restriction enzyme recognition sequence adjacent to the unique tag-sequence. Specific cleavage within the tag-sequence motif is obtained by rendering the recognition sequence double-stranded with an oligonucleotide having a sequence general for all nucleic acids and/or a pool of random short

oligonucleotides, e.g. hexamers, that will form double-stranded substrates for restriction cleavage.

The present invention should also be of value for synthesis of oligonucleotides. Chemically synthesised oligonucleotides are usually contaminated by a large fraction of shorter oligonucleotides that are difficult to remove by purification. These incomplete products contribute to a decreased efficiency in e.g. templated ligation reactions. However, oligonucleotides produced according to the present invention will per definition be devoid of sequences that cannot circularise by templated ligation. Therefore the oligonucleotides can be synthesised from circularised templates, generated from a crude chemical synthesis where even a minority of the products are of full length, since only products of sufficient integrity will circularise. These will template the synthesis of oligonucleotides in yields thousand fold greater than the initial chemical synthesis scale. The synthesis of oligonucleotides may be performed through 1, 2 or more generations of RCA as described above. The concatemerised oligonucleotide sequences could be specifically monomerised at a defined position, e.g. by using the above mentioned general cleavage procedure using a type IIs restriction enzyme and random short oligonucleotides. Excess random short oligonucleotides, as well as other components of the enzymatic buffers, can be removed from the monomerised oligonucleotide synthesis by size separation using chromatography matrices or electrophoresis, or by affinity purification of either tagged probes or random oligonucleotides. This oligonucleotide synthesis procedure should be of particular importance for synthesis of long oligonucleotides of high integrity used as e.g. padlock probes or aptamers.

Repeated RCAs has been proposed previously in a WO 92/01813. However, in WO 92/01813 the RCA products are cleaved down to monomers in order to create new primers for subsequent RCA reactions using preformed circles. Japanese patent application, JP 4-304900, describes using RCA for detecting circularised probes. In this application repeated RCAs are described using the RCA product as a target for subsequent probe ligations and RCAs. The present invention uses the monomerised RCA product as probe in

subsequent ligations and RCAs. The advantages of this procedure have already been discussed.

In a further aspect of the invention, the present inventors have devised several approaches for increasing the signal obtained from an RCA by adding a linear signal-generating amplification of the RCA product. This result in an amplification of the signal from circularised probes that grow as a square function of time.

The first approach is based on selective, hybridisation dependent, degradation of a probe complementary to the RCA product. It is desirable that the probe is designed in a manner such that its cleavage is detectable.

Accordingly, in a further aspect, the invention comprises a method of nucleic acid amplification which employs probes to indicate the extent of the amplification, which method comprises the steps of:

- providing a signaling probe, which probe includes a sequence which is complementary to an amplification product;

- reacting the signaling probe with the amplification product;

- selectively degrading signaling probes that have hybridised to the first generation amplification product, wherein degraded probes dissociate from the first generation amplification product allowing further signaling probes hybridise with the product, wherein hybridisation and degradation of the probes effects a change in signal emitted by the probe.

Preferably, the probe consists of a hairpin-loop probe, a so-called molecular beacon (Tyagi & Kramer, 1996) comprising a detectable marker ligand therein, which selectively emits a detectable signal. The marker may be part of the nucleic acid sequence or may be a ligand held within the hairpin formation. Preferably, the ligand is released from the nucleic acid on cleavage of the nucleic acid.

If the part of the probe hybridising to the RCA product at least partially consists of RNA it will, upon hybridisation, be degraded by the enzyme RNase H. (Duck et al, e.g. USP5011769, USP5660988, USP5403711 and USP6121001)

A similar effect can be obtained by substituting one or more of the deoxynucleotides, used in the RCA, for thiophosphorodeoxynucleotides, thereby protecting the RCA product from endonuclease hydrolysis. The molecular beacon would in this case be made of ordinary deoxynucleotides and would therefore be susceptible to degradation by a double strand specific endonuclease. Specifically, a restriction enzyme recognition site can be designed into the loop sequence of the beacon. There are several known restriction enzymes that specifically cleave the non-thiophosphorylated strand when hybridised to a thiophosporylated strand. This would, by analogy to the RNaseH degradable probe, only result in cleavage and thereby signal of the probe in the presence of the target molecules. Instead of a specific endonuclease, any unspecific double strand specific endonuclease can be used, thereby avoiding the need for a specific sequence in the probe.

A related way of producing detectable cleavage products is to add a probe complementary to a RCA product and a restriction enzyme that recognises a sequence in the RCA-oligonucleotide duplex. After cleavage of the RCA-oligonucleotide duplex, the cleaved probe will dissociate from the monomerized RCA products. Then an intact oligonucleotide can hybridise to one end of the cleaved monomer, whereafter the other end of the monomer will most likely hybridise to the same oligonucleotide, due to the intra-molecular nature of the interaction. In this manner, a new substrate for the restriction enzyme is formed, and a second signal-generating cleavage occurs. By labelling the probe with a fluorescent moiety at one end and a quencher molecule at the other end a signal will be generated upon cleavage and subsequent dissociation.

Hybridisation-dependent degradation of probes can also be performed with any double strand-specific exonuclease ((Copley & Boot, 1992), US6121001). In this case a probe that hybridise to the RCA product will be a

substrate for the exonuclease and thereby degraded while the RCA product is unaffected due to the lack of any double stranded ends. Upon degradation the melting temperature of the duplex will decrease and the probe is more likely to dissociate even if not fully degraded. Thereby the sequence is made accessible for another probe to hybridise and subsequently be degraded.

This approach for increasing the signal can also be performed with ribo- or so called DNA-zymes (R/D-zyme) (e.g. (Herschlag & Cech, 1990) & (Carmi *et al.*, 1998). By incorporating the complementary sequence of an R/D-zyme in the circularised nucleic acid the RCA product will contain the active R/D-zyme. This can then be used to cleave a probe that is added to the reaction mixture. Upon cleavage the cleaved probe will dissociated from the RCA product and leave place for another uncleaved probe to hybridise and be cleaved. The R/D-zyme does not need to cleave the probe but can instead ligate ((Cuenoud & Szostak, 1995)) two oligonucleotides together and generate a signal as described below.

Probes may also be designed so that upon hybridisation to the RCA product they assemble in such a way that they resemble the substrate for a structure specific enzyme. The enzyme is preferably selected from the group comprising resolvases, recombinases or nucleases, examples Ruv ABC, Holliday junction resolvases, Flip recombinases, FEN nuclease or certain polymerases. One aspect of the invention is to use two oligonucleotides hybridising in tandem in such a way that the downstream oligonucleotide has a protruding 5 prime end. This structure can be cleaved by several different enzymes e.g. FEN nucleases, *Taq* polymerase, *Tth* polymerase or *Mja* nuclease ((Lyamichev *et al.*, 1999). The downstream oligonucleotide is designed so that it has a melting temperature near the temperature of the isothermal RCA reaction.

Accordingly, the probes may comprise a fluorescent moiety and a quenching moiety which are separated by a hairpin loop structure, wherein in an un-bound and intact conformation the quenching moiety quenches the signal from the fluorescent moiety, and wherein bound or degraded probe emits a signal. Alternatively, the probe may include a pair of signalling

moieties, which moieties produce a signal by FRET when the probe is intact, wherein degradation of the probe inhibits signal production.

Advantageously, with the above combination of a probe with any of the hybridization selective degradable features the reaction can continue isothermally, even at low temperature, and be detectable.

In this manner the temperature may be less than 60°C and preferably is less than 50°C. Advantageously, operating at these temperature removes the need to maintain the reactions at elevated temperature.

Instead of degrading probes it is possible to assemble probes to mark the presence of an RCA product. If two oligonucleotide probes are constructed in such a way that they hybridise adjacent to each other on the RCA product they can subsequently be joined by a ligase. If the two oligonucleotides are designed in such a way that upon ligation and dissociation from the target they form a stable stem-loop structure, a donator molecule at the 5 prime position can be placed in close proximity to a acceptor molecule at the 3 prime position in the other oligonucleotide. A signal can then be generated based upon fluorescence resonance energy transfer (FRET). By designing the probes so that the ligated probe will have a melting temperature near the temperature of the isothermally proceeding RCA reaction a fast turnover will be possible. The unligated probes will be present in large molar excess over the produced ligated probes, thereby increasing the rate at which they will hybridise to the target RCA product and subsequently be ligated, dissociate and so on. The reaction is thus possible to perform isothermally.

Accordingly, in a further aspect, there is provided a method of nucleic acid amplification which employs probes to indicate the extent of the amplification, which method employs a pair of signaling probes, which probes are designed such that they hybridise to a target sequence on an amplification product adjacent to each other, wherein upon hybridisation the probes are ligated to form a ligated product which dissociates from the target sequence, wherein dissociation of the ligated product from the target sequence allows a further pair of probes hybridise to the target sequence, and wherein upon dissociation from the target sequence the ligated product emits a signal.

Preferably, one of the pair of signaling probes comprises a donor moiety and an other comprises an acceptor moiety, and wherein the probes are designed such that the ligated product forms a hairpin loop structure, which upon formation allows energy transfer between the donor and acceptor moieties to produce a signal.

In a yet further aspect, the present inventors have also found that molecular beacons generate signal in absence of RCA product but in presence of DNA polymerase, unless at least parts of the DNA residues in the beacon is replaced by nucleic acid residues that are not accepted as templates/substrates for the polymerase. Whilst being bound by theory, there are two possible explanations for the phenomenon. Either the 3' end (usually carrying the fluorescence quencher) is degraded by the 3' exonucleolytic (proof reading) activity of the polymerase, or the structure is opened-up because of enzyme binding. Nevertheless, generation of non-specific signal can be completely avoided by using molecular beacons comprising 2'-O-methyl-RNA residues instead of DNA residues. Furthermore, molecular beacons that hybridise to an RCA product are quenched, unless the molecular beacons are designed not to form inter-molecular structures on the RCA product. When the beacons hybridise to the RCA products the stem part of the beacon is opened, bringing the fluorophore and quencher apart. This should produce a fluorescence signal from the non-quenched fluorophore. However, neighbouring beacons readily form stems with each other (inter-molecular stems), bringing the quencher and reporter fluorophores close together, much like the closed (quenched) conformation of non-hybridising beacons (Fig. 5). This can be avoided by including one of the stem sequences of the beacon in the padlock probe sequence so that, upon hybridisation to the RCA product, one part of the stem hybridises to the product so that it cannot form stem-like structures with neighbouring beacons (Fig. 5). With these modifications the RCA can be monitored in real-time (Fig. 6). Alternatively, a second probe could be added to the detection reaction that hybridise to the RCA product in between the molecular beacons to avoid that this sequence loops out, thus hindering the inter-molecular stems to form. The

magnitude of the inter-molecular quenching may vary between different molecular beacon sequences as exemplified by the complete quenching of the DNA molecular beacon version (Fig. 7) compared to the partial quenching of the 2'-O-Me-RNA beacon used in figure 6.

The inter-molecular hairpin structure that forms between adjacent molecular beacons, hybridising to a concatenate sequence, can also be used to generate FRET between two molecular beacons that hybridise within one monomer of the concatenate sequence. The two molecular beacons should be equipped with the same stem sequence, but the position of the quencher and fluorophore should be switched in one of the two molecular beacons. Such molecular beacons will hybridise in an alternating fashion, forming inter-molecular hairpin structures between adjacent, but alternating molecular beacons, such that the fluorophore of one beacon is positioned in close proximity to the fluorophore of the neighbouring molecular beacon, enabling efficient FRET between the two fluorophores. In this way background fluorescence from closed molecular beacons can be diminished, increasing detection sensitivity.

To enable the present enhanced detection procedures of RCA products it is prerequisite that the initial RCA product is single-stranded. This in turn requires the elimination of non-circularised probes that remain after the first ligation reaction, because they can hybridise to the initial RCA product and prime synthesis of the complementary strand.

Moreover, it has been noticed that remaining non-circularised probes produce a non-specific signal in RCAs. An attempt to overcome this problem is described in WO 00/36141 where enzymes or so-called capture ligands are used to remove any remaining linear nucleic acid probes.

Accordingly, in a further aspect, the present invention relates to a method of removing or rendering inert non-circularised probes during (or after) a nucleic acid amplification process which utilises circular probes, in which the non-circularised probes comprise first and second segments separated by a linking segment, wherein the first and second segments are complementary to sequences on a target sequence, wherein the probe is designed to form a

hairpin loop structure between the 3' end of the probe and a sequence in a linking segment of the probe, wherein a stem of the hairpin loop structure ideally has a thermal stability that neither inhibits formation of a hybrid between the loop and the target sequence nor inhibit replication of the probe by RCA.

The stem should be sufficiently stable to prime synthesis and thus convert non-ligated probes to full hairpins, unable to prime "second strand" synthesis on the RCA-product.

Additionally, the hairpin-forming probe has a further advantage in that such a probe design may be more specific than conventionally designed probes. In conventional probe design the diagnostic base should be positioned at the ultimate 3' position of the probe sequence to fully take advantage of the mismatch discriminatory capacity of DNA ligases. In the hairpin probes of the present invention, this diagnostic base will thus be a part of the hairpin forming sequence. This sequence may be designed to flip back and forth between the hairpin and the target hybridising conformation at the ligation temperature. The matched sequence version of a probe will then spend more time in the target sequence hybridising conformation than the corresponding mismatched probe version. This will favour ligation of matched probes over misligation of mismatched probes.

In a further aspect, the invention provides a further method of removing or rendering inert non-circularised probes during (or after) a nucleic acid amplification process which utilises circular probes, in which an excess of oligonucleotides which are complementary to a 3' end of the probes are added before the amplification reaction, which oligonucleotides preferably include a 5' sequence extension, whereby the 3' end of non-circularised probes will lose complementarity to a product of the amplification process

Preferably, the excess of oligonucleotides which are complementary to the 3' end of the probes are added to the system before the RCA is begun although they may be added simultaneously with the other reagents or immediately after the reaction is begun.

According to the invention, the 3' ends of non-ligated probes will lose their complementarity to the RCA product, and will not be able to prime a second strand synthesis. These 3' eliminators may be used to prime the RCA.

Alternatively, to avoid the situation where the linking segment of non-ligated probes becomes replicated, the 3' eliminators may be equipped with an unextendable 3' end that is also unremovable by 3' exonucleolytic activities of DNA polymerases. This may be particularly important if the RCA product is intended to be detected by oligonucleotides recognizing sequences in the RCA product that correspond to the linking segment of the padlock probes. In this case a general primer could be added to the circularised probes to initiate the RCA. Because of the limited length of the 3' eliminators, they will become displaced by the polymerase extending the primer and will thus not inhibit the RCA (Banér *et al.*, 1998).

Detailed Description of the Invention

Embodiments of the invention will now be described in more detail, by way of example only, with reference to the accompanying drawings of which:

Figure 1 illustrates the general procedure of several generation rolling circle replication (RCA) according to the invention;

Figure 2 shows real time monitoring of third generation RCA;

Figure 3 shows the fluorescence recorded at a microarray feature containing an oligonucleotide complementary to the second-generation RCA product obtained according to example 2.

Figure 4 is a graph showing removal of non-specific accumulation of fluorescence in presence of DNA polymerase by replacing all DNA residues of the molecular beacon with 2'O-Me-RNA residues where one DNA molecular beacon is labelled with FAM fluorescence (upper panel) and one 2'O-Me-RNA molecular beacon is labelled with HEX fluorophore

(lower panel) which was added to the same test tube in presence (squares) or in absence (circles) of ϕ29 DNA polymerase;

Figure 5 is a schematic representation of inter-molecular quenching of molecular beacons hybridizing to an RCA product when using a traditional molecular beacon design;

Figure 6 is a graph showing the inter-molecular beacon quenching is demonstrated by restriction cleavage of the RCA product, and the modified design of the molecular beacon allows for real-time monitoring of RCA;

Figure 7 is a graph showing the temperature profile of RCA reactions performed as in figure 6. Fluorescence is obtained from the DNA molecular beacon added after RCA, and heat inactivation of the polymerase, and

Figure 8 is a schematic representation of the amplification method of the invention whereby molecular beacons are degraded.

EXAMPLES

Example 1 (Real-Time Monitoring of a Third-Generation RCA Using Modified Molecular Beacons)

2 nM padlock probe (P-CCTCCCATCATATTAAAGGCTTTCTCTATGTTAAGTGACCTACGACCTCA ATGCTGCTGCTGTACTACTCTTCCTAAGGCATTCTGCAAACAT (P= 5' phosphate) was circularised in 10 mM TrisAc (pH 7.5), 10 mM $MgAc_2$, 50 mM KAc, 0,1% BSA, 1mM ATP, 200mM NaCl, and 20 mU/μl T4 DNA ligase in presence of 0, 10, 40, or 160 zmol of a target oligonucleotide (GCCTTTAATATGGGAGGATGTTTGCAGAATGCCTTAG). The reactions were incubated for 15 minutes at 37^0C, and then the ligase was inactivated for

5 minutes at 65^{0}C. The first-generation RCA was performed for 45 minutes at 37^{0}C in 0,1μg/μl BSA, 250μM dNTP, 10 mM DTT, 1 pmol primer (CGTCGTAGGTCACTTAACAT), and 1ng/μl ϕ29 DNA polymerase. The polymerisation components were added to 10 μl ligation reactions in 15 μl of ϕ29 DNA polymerase buffer (10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 20 mM $(NH_4)_2SO_4$). The DNA polymerase was inactivated for 10 min at 65^{0}C. The first-generation RCA product was monomerised by adding 5 μl 0,1 μg/μl BSA, 3 pmol RSAI (GCTGCTGTACTACTCTCTT), and 10 U *Rsa*I in ϕ29 DNA polymerase buffer. The reaction was incubated for 60 minutes at 37^{0}C and then the enzyme was inactivated for 10 minutes at 65^{0}C. The monomerised RCA product was circularised by adding 5 μl 0.1 μg/μl BSA, 1 mM ATP, and 1 U T4 DNA ligase in ϕ29 polymerase buffer. The ligation was incubated for 15 min at 37^{0}C and then the enzyme was inactivated for 5 minutes at 65^{0}C. The second-generation RCA was performed using the same conditions as the first-generation RCA by adding 15 μl polymerisation reagents to 35 μl of the circularised RCA product. The polymerisation reaction continued for 45 minutes at 37^{0}C. Half of the second-generation RCA product was monomerised by adding 6 pmol RSAIcomp (AAGAGAGTAGTACAGCAGC) and 10 U *Rsa*I in 5 μl ϕ29 DNA polymerase buffer including 0.1 μg/μl BSA. The reaction was incubated for 60 minutes at 37^{0}C, and then the enzyme was inactivated for 10 minutes at 65^{0}C. Circularisation of the monomerised second-generation RCA product was performed using the same procedure as the circularisation of the monomerised first-generation RCA product. The third-generation RCA was performed as the second-generation RCA but in presence of 0.1 μM molecular beacon (HEX-ccucAAUGCUGCUGCUGUACUACgagg-DABCYL) and for 60 min. The reaction was followed in real-time using a ABI 7700.

Example 2, (Detection of a second-generation RCA product on a DNA microarray)

2 nM padlock probe (WD 1216G) was circularised as above in presence of various amounts of target oligonucleotides (T 1216G; 0, 25, 250, or 2500 zmol). The first-generation RCA was performed for 100 minutes at 37^{0}C as above 1 pmol of the primer WDP-F. The polymerisation components were added to 10 μl ligation reactions in 15 μl of ϕ29 DNA polymerase buffer. The DNA polymerase was inactivated for 10 min at 65^{0}C. The first-generation RCA product was monomerised by adding 5 μl 0,1 μg/μl BSA, 3 pmol Comp WDP-F, and 5U *Fnu*4H 1 in ϕ29 DNA polymerase buffer. The reaction was incubated for 60 minutes at 37^{0}C and then the enzyme was inactivated for 10 minutes at 65^{0}C. The monomerised RCA product was circularised by adding 5 μl 0.1 μg/μl BSA, 1 mM ATP, and 1 U T4 DNA ligase in ϕ29 polymerase buffer. The ligation was incubated for 15 min at 37^{0}C and then the enzyme was inactivated for 5 minutes at 65^{0}C. The second-generation RCA was performed using the same conditions as the first-generation RCA by adding 15 μl polymerisation reagents to 35 μl of the circularised RCA product. The polymerisation reaction continued for 100 minutes at 37^{0}C. Half of the second-generation RCA product was monomerised by adding 4.5 pmol D-RCRcut and 5 U *Fnu*4HI in 5 μl ϕ29 DNA polymerase buffer including 0.1 μg/μl BSA. The reaction was incubated for 60 minutes at 37^{0}C, and then the enzyme was inactivated for 10 minutes at 65^{0}C. 30μl monomerised second-generation RCA product was hybridised to a DNA microarray in 4*SSC, 0,525 μM Comp WD Cy5, 10μM EDTA at 45^{0}C for 2h, washed in 0.1xSSC at 45^{0}C, rinsed in water, and finally dried. The Cy5 fluorescence signal was recorded in a fluorescence laser scanner.

A schematic drawing of the general procedure of a several generation RCA, as described in examples 1 and 2 is illustrated in Figure 1. The results of examples 1 and 2 are shown in figures 2 and 3, respectively. First a circular nucleic acid is replicated in an RCA. Then the first-generation RCA product is monomerised, e.g. by using a restriction enzyme that will cleave the product at a recognition site, rendered double-stranded by an oligonucleotide complementary to the RCA product. Intact restriction oligonucleotides will

displace the digested ones, e.g. during or after heat inactivation of the restriction enzyme. When an intact restriction hybridises to one end of a monomerised RCA product, the other end of the monomer will hybridise to the same restriction oligonucleotide, because of the intra-molecular nature of the second hybridisation reaction. The monomers can then be circularised by joining the ends, e.g. using a DNA ligase. The procedure can now be repeated for one or more rounds of the same procedure.

Figure 2 shows real-time monitoring of the third-generation RCA described in example 1. **A)** Real-time measurement of HEX fluorescence emitted from molecular beacons hybridising to the RCA product as it is generated. **B)** A graph showing the relationship between the amount of target oligonucleotide added in the first probe circularisation reaction, performed in quadruplicate, and the maximum slope of the third-generation real-time RCA of these ligation reactions. The error-bars denote the standard deviation.

The fluorescence recorded at a microarray feature containing an oligonucleotide complementary to the second-generation RCA product obtained according to example 2.

Example 3

Oligonucleotides: The padlock probes used were p90: P-CCTCCCATCATATTAAAGGCTTTCTCTATGTTAAGTGACCTACGACGATG CTGCTGCTGTACTACTCTTCCTAAGGCATTCTGCAAACAT and p93: P-CCTCCCATCATATTAAAGGCTTTCTCTATGTTAAGTGACCTACGACCTCA ATGCTGCTGCTGTACTACTCTTCCTAAGGCATTCTGCAAACAT (P= 5' phosphate). The ligation template for the padlock probes was t40: GCCTTTAATATGGGAGGATGTTTGCAGAATGCCTTAG. The DNA molecular beacon was FAM-cgcctcAATGCTGCTGCTGTACTACgaggcg-DABCYL (the stem part in lower case) and the 2' O-Me-RNA molecular beacon was HEX-ccucAAUGCUGCUGCUGUACUACgagg-DABCYL. The stem is two base pairs shorter in the 2'-O-Me-RNA beacon because of the higher hybrid stability of 2'-O-Me-RNA base pairs. The oligonucleotide used

for restriction digestion was Tsp45I: GGCTTTCTCTATGTTAAGTGACCTACGA.

Example 4

Padlock probe circularization: 200 nM padlock probes were ligated in 10 mM Tris-acetate pH 7.5, 10 mM MgAcetate, 50 mM NaCl, 1 mM ATP, 1 µg/µl BSA, and 0.2 units/µl T4 DNA ligase (Amersham Pharmacia Biotech) at 37°C for 30 minutes in presence of 600 nM ligation template.

Example 5

Rolling-circle amplification: Polymerization reactions were performed in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 10 mM dithiothreitol and 0.2 µg/µl BSA, 0.25 mM dNTP, and 2 ng/µl Φ29 DNA polymerase (kindly provided by Dr. M. Salas) at 37°C. For real-time monitoring the RCA was performed in presence of 100 nM molecular beacon and 300 nM ROX dye. Fluorescence values are given as a ratio between the fluorescence emitted by the molecular beacon (FAM or HEX) and the ROX reference dye. The temperature profiles were obtained by sampling fluorescence after temperature increments of 1°C held for 30 seconds.

Example 6

Restriction digestion: 20 µl of a 10 mM Bis Tris Propane-HCl (pH 7.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 µg/µl BSA, 1,5 µM Tsp45I, and 0.1 U/µl Tsp 45I (New England Biolabs) was added to 40 µl RCA products and incubated at 65°C for four hours.

Turning to the drawings as can be seen in Figure 1 removal of non-specific accumulation of fluorescence in presence of DNA polymerase by replacing all DNA residues of the molecular beacon with 2'O-Me-RNA residues. One DNA molecular beacon labelled with FAM fluorescence (upper panel) and one 2'O-Me-RNA molecular beacon labelled with HEX fluorophore (lower panel) was added to the same test tube in presence (squares) or in absence (circles) of Φ29 DNA polymerase. The left portion of the graphs

shows a real time monitoring of fluorescence in the test tube, and the right portion shows the temperature profile of the components present at the end of the 60 min incubation.

From Figure 2 it can be seen that inter-molecular quenching of molecular beacons hybridizing to an RCA product when using a traditional molecular beacon design (upper panel). The structure can be avoided by using a modified design (lower panel).

The inter-molecular beacon quenching shown in figure 3 is demonstrated by restriction cleavage of the RCA product, and the modified design of the molecular beacon allows for real-time monitoring of RCA. RCA was performed on ligation reactions subjected to ligase (black) or no ligase (grey) containing either the p90 (squares) or the p93 (circles) padlock probes. The left portion of the graph shows a real time monitoring of fluorescence from the 2' O-Me-RNA molecular beacon in the different reactions. The right portion shows the temperature profile of the components present at the end of the 90 min RCA (filled symbols). Superimposed are the temperature profiles of the different reaction components after a restriction digest (open symbols).

Temperature profile of RCA reactions performed as in figure 3 are shown in figure 4 where fluorescence is obtained from the DNA molecular beacon added after RCA, and heat inactivation of the polymerase.

The invention is not limited to the embodiments hereinbefore described which may be varied in both construction and detail without departing from the spirit of the invention.

References

Banér J., Nilsson M., Mendel-Hartvig M., and Landegren U. (1998). Signal amplification of padlock probes by rolling circle replication. *Nucleic Acids Res.* **22:** 5073-5078.

Carmi N., Balkhi S. R., and Breaker R. R. (1998). Cleaving DNA with DNA. *Proc Natl Acad Sci U S A* **95:** 2233-7.

Copley C. G., and Boot C. (1992). Exonuclease cycling assay: an amplified assay for the detection of specific DNA sequences. *Biotechniques* **13:** 888-92.

Cuenoud B., and Szostak J. W. (1995). A DNA metalloenzyme with DNA ligase activity. *Nature* **375:** 611-4.

Herschlag D., and Cech T. R. (1990). DNA cleavage catalysed by the ribozyme from Tetrahymena. *Nature* **344:** 405-9.

Lizardi P. M., Huang X., Zhu Z., Bray-Ward P., Thomas D. C., and Ward D. C. (1998). Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nature Genet.* **19:** 225-232.

Lyamichev V., Mast A. L., Hall J. G., Prudent J. R., Kaiser M. W., Takova T., Kwiatkowski R. W., Sander T. J., de Arruda M., Arco D. A., Neri B. P., and Brow M. A. D. (1999). Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. *Nat. Biotechnol.* **17:** 292-296.

Tyagi S., and Kramer F. R. (1996). Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.* **14:** 303-308.

CLAIMS

1. A method of amplifying a nucleic acid product, comprising the steps of:

   - providing a first generation amplification product, which product comprises a concatamer of a sequence to be amplified;
   - monomerising the amplification product; and
   - carrying out a further amplification of the thus-formed monomers to form a second generation amplification product.

2. A method as claimed in claim 1 in which the monomers are ligated to form circles prior to further amplification of the circles.

3. A method as claimed in any claim 2 in which the first generation amplification product is a linear rolling circle amplification (RCA) product.

4. A method as claimed in claim 3 in which the further amplification is RCA.

5. A method according to any preceding claim in which the sequence to be amplified is a probe sequence.

6. A method according to any preceding claim in which the sequence to be amplified comprises cDNA, genomic DNA or RNA sequences.

7. A method as claimed in any preceding claim in which monomerisation of the first generation amplification product is achieved using a restriction enzyme and an oligonucleotide complementary to the first generation amplification product, wherein the restriction enzyme cleaves any first generation amplification product/oligonucleotide hybrids.

8. A method as claimed in claim 5 in which oligonucleotide is added in excess to a number of monomers contained in the first generation reaction product.

9. A method as claimed in any of claims 1 to 7 in which the sequence to be amplified includes a catalytically active sequence which enables monomeristaion of the first generation amplification product.

10. A method as claimed in any previous claim in which the first generation amplification product is produced in a first generation amplification step, which step utilises a polymerase enzyme, wherein the method includes an initial step of inactivating the polymerase enzyme.

11. A method as claimed in any preceding claim carried out in isothermal conditions.

12. A method as claimed in any of claims 2 to 11 in which circularised amplification products are hybridised to different primers attached to a solid support, which primers initiate localised RCA.

13. A method as claimed in claim 12 in which the primers are zip-code or tag sequences.

14. A method of nucleic acid amplification which employs probes to indicate the extent of the amplification, which method comprises the steps of:

- providing a signaling probe, which probe includes a sequence which is complementary to an amplification product;
- reacting the signaling probe with the amplification product;

- selectively degrading signaling probes that have hybridised to the first generation amplification product, wherein degraded probes dissociate from the first generation amplification product allowing further signaling probes hybridise with the product, wherein hybridisation and degradation of the probes effects a change in signal emitted by the probe.

15. A method as claimed in claim 14 in which the probe includes a sequence which is susceptible to degradation when the probe has hybridised to the amplification product.

16. A method as claimed in claims 14 or 15 in which the probe, and especially the sequence of the probe which is complementary to the amplification product, includes a sequence of RNA residues which is susceptible to degradation by a suitable enzyme such as RnaseH when the probe has hybridised to the amplification product.

17. A method as claimed in claims 14 or 15 in which the probe, when bound to the amplification product, is degraded by endonucleases, wherein the amplification product is modified to prevent degradation by the endonuclease.

18. A method as claimed in claim 17 in which the amplification product is modified by substituting thiophosphorodeoxynucleotides for deoxynucleotides.

19. A method as claimed in claims 17 or 18 in which a restriction enzyme recognition site is included in the sequence of the probe which hybridises with the first generation amplification product.

20. A method as claimed in claims 14 or 15 in which selective degradation of the probe is achieved by means of a double strand specific exonuclease.

21. A method as claimed in claims 14 or 15 in which selective degradation of the probe is achieved using DNA-zymes, which DNA-zymes are contained in the first generation amplification product.

22. A method according to any of claims 14 to 21 in which the probes comprise a fluorescent moiety and a quenching moiety which are separated by a hairpin loop structure, wherein in an un-bound and intact conformation the quenching moiety quenches the signal from the fluorescent moiety, and wherein bound or degraded probe emits a signal.

23. A method according to claim 22 in which at least one of the stem sequences of the hairpin loop of the probe is designed to hybridise with the amplification product.

24. A method as claimed in any of claims 14 to 21 in which the probe includes a pair of signalling moieties, which moieties produce a signal by FRET when the probe is intact, wherein degradation of the probe inhibits signal production.

25. A method according to any of claims 14 to 24 in which the probes are designed such that they will not act as templates/substrates for DNA ploymerase.

26. A method according to any of claims 14 to 25 in which the amplification product is a single stranded linear RCA product.

27. A method as claimed in any of claims 14 to 26 in which dissociation of the degraded probe from the amplification product is achieved by thermal cycling.

28. A method as claimed in any of claims 14 to 27 in which the signaling probe is added during or after nucleic acid amplification.

29. A method of nucleic acid amplification which employs probes to indicate the extent of the amplification, which method employs a pair of signaling probes, which probes are designed such that they hybridise to a target sequence on an amplification product adjacent to each other, wherein upon hybridisation the probes are ligated to form a ligated product which dissociates from the target sequence, wherein dissociation of the ligated product from the target sequence allows a further pair of probes hybridise to the target sequence, and wherein upon dissociation from the target sequence the ligated product emits a signal.

30. A method as claimed in claim 29 in which one of the pair of signaling probes comprises a donor moiety and an other comprises an acceptor moiety, and wherein the probes are designed such that the ligated product forms a hairpin loop structure, which upon formation allows energy transfer between the donor and acceptor moieties to produce a signal.

31. A method according to any of claims 2 to 31 in which, as an initial step, any non-circularised probes are removed, or rendered inert.

32. A method according to claim 31 in which non-circularised probes are removed using exonucleases.

33. A method according to claim 31 in which non-circularised probes are removed by capture on a solid support carrying an appropriate ligand for the non-circularised probes.

34. A method as claimed in claim 34 in which the ligand is a nucleic acid sequence which has sequence affinity for non-circularised probe.

35. A method as claimed in claim 31, wherein the non-circularised probes comprise first and second segments separated by a linking segment, wherein the first and second segments are complementary to sequences on a target sequence, wherein the probe is designed to form a hairpin loop structure between the 3' end of the probe and a sequence in a linking segment of the probe, wherein a stem of the hairpin loop structure ideally has a thermal stability that neither inhibits formation of a hybrid between the loop and the target sequence nor inhibit replication of the probe by RCA.

36. A method as claimed in claim 31 in which an excess of oligonucleotides which are complementary to a 3' end of the probes are added before the amplification reaction, which oligonucleotides preferably include a 5' sequence extension, whereby the 3' end of non-circularised probes will lose complementarity to a product of the amplification process.

37. A method of removing or rendering inert non-circularised probes during (or after) a nucleic acid amplification process which utilises circular probes, in which the non-circularised probes comprise first and second segments separated by a linking segment, wherein the first and second segments are complementary to sequences on a target sequence, wherein the probe is designed to form a hairpin loop structure between the 3' end of the probe and a sequence in a linking segment of the probe, wherein a stem of the hairpin loop structure ideally has a

thermal stability that neither inhibits formation of a hybrid between the loop and the target sequence nor inhibit replication of the probe by RCA.

38. A method of removing or rendering inert non-circularised probes during (or after) a nucleic acid amplification process which utilises circular probes, in which an excess of oligonucleotides which are complementary to a 3' end of the probes are added before the amplification reaction, which oligonucleotides preferably include a 5' sequence extension, whereby the 3' end of non-circularised probes will lose complementarity to a product of the amplification process.

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Databases searched:

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UK Cl (Ed.T):

Int Cl (Ed.7): C12Q 1/68

Other: ONLINE: WPI, EPODOC, JAPIO, MEDLINE, EMBASE, BIOSIS, SCISEARCH, CAPLUS

Documents considered to be relevant:

Category	Identity of document and relevant passage		Relevant to claims
A	WO92/01813 A1	(SYNGENE, INC.) See especially, page 16 line 23- page 17 line 3, page 20 line 23- page 21 line 2 and examples	
A	US5854033 A	(LIZARDI) See especially column 19 line 21- column 21 line 21, column 23 line 53- column 24 line 40 and examples	
A	*Biotechniques*; Vol 30 (3), pp 584-588 (2001). Myer & Day. See especially Results and Discussion		
A	*Nucl Acid Res*; Vol 28 (12), pp E58 (2000). Antson *et al.* See especially Discussion		
A	*Nucl Acid Res*; Vol 26 (22), pp 5073-5078 (1998). Banér *et al.* See especially Discussion		

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
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