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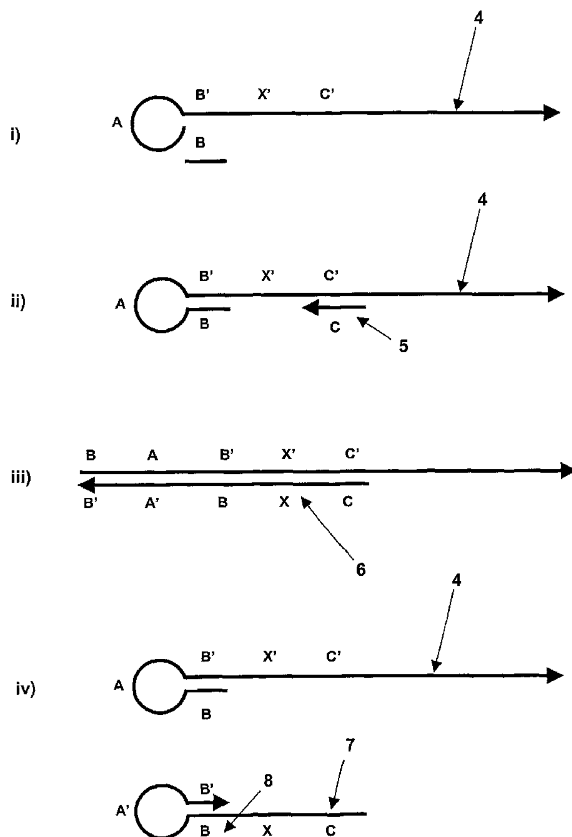
(54) Title: AMPLIFICATION OF NUCLEIC ACIDS



(57) Abstract: A method of amplifying a nucleic acid sequences comprises the steps of providing a reaction mixture comprised of: (i) providing a reaction mixture comprised of: (a) a polymerase enzyme system including a strand displacing polymerase; (b) a target nucleic acid strand incorporating the sequence to be amplified; (c) a first primer having a 3' region capable of hybridising to the target nucleic acid strand and copying said sequence as a template to form a first primer extension product, and a 5' region capable of self-hybridising to said first primer extension product to form a first single stranded loop structure; and (d) a second primer capable of being hybridised to the target strand to copy the target strand back towards the loop to form a second primer extension product which is capable of self-hybridisation to form a second single stranded loop structure to which said first primer is capable of being hybridised and then extended; and; (ii) effecting repeated cycles of hybridisation, polymerisation and denaturation to effect amplification of said sequence.

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Amplification of Nucleic Acids

Field of Invention

The present invention relates to the amplification of nucleic acids and their natural and chemical analogues.

Background of the Invention

PCR was the first, and is still most widely used, method to amplify the numbers of a desired DNA sequence in an exponential manner without concomitant amplification of background DNA. The process made possible the study of DNA from samples of a complexity and concentration which would otherwise be practically impossible other than by, say, cloning.

The technique relies upon the separation of the strands of a double stranded target DNA molecule and the hybridisation to each of oligonucleotide primers from which polynucleotide synthesis may be initiated. These primers are arranged 'face to face' such that the direction of DNA synthesis of each is toward the location of the other.

After synthesis of two new DNA strands from these primers the strands are again denatured and more primers allowed to bind for yet further rounds of synthesis and denaturation. By cycling this reaction it can be demonstrated that exponentially increasing numbers of DNA fragments are produced whose sequences span the target double strand between the sites of the two primers.

A number of other amplification techniques have been developed since the invention of PCR and these can generally be divided into those which employ thermocycling, or some other means of cyclic strand denaturation (such as electric charge or sonication (Clarke and Minton patent ref), and those which perform isothermally and regenerate primer sites by some other method and both of these types have their advantages and disadvantages.

Among the amplification techniques that seek to amplify their target without the need to separate the target strands is Strand displacement amplification (SDA) in which a DNA polymerase is used whose properties are such that it displaces the strand ahead of it when polymerising along a double stranded section of DNA. This contrasts with the type of DNA polymerase conventionally used for PCR, which would degrade a downstream strand upon contact. SDA involves the use of two primers carrying cleavage sites for a restriction enzyme that can be protected from cutting by the incorporation of modified nucleotide bases in such a way that only the unmodified strand is cleaved by the enzyme. The reaction is preformed in the presence of a dNTP carrying this modification such that newly synthesised material is protected from digestion while the primers themselves are not. Thus, the primers undergo cycles of restriction 'nicking' and extension by strand displacement and exponential amplification of the sequence between the primers occurs.

Another isothermal exponential amplification technique is Rolling Circle Amplification (RCA) (Lizardi - US5854033). In this amplification technique a covalently closed circle of DNA acts as a template for a linear oligonucleotide primer. This primer is extended by a strand displacing DNA polymerase and proceeds round the circle, displacing downstream sequences as it goes to create a string of covalently joined copies of the complement to the circular temple. A second oligonucleotide primer, designed to hybridise to a specific point on this complementary strand extends to make copies of this strand. These copies are displaced by yet further copies made upstream of their hybridisation site. These displaced copies then act as substrates for the hybridisation of the original primer and an exponential generation of copies of the circle and its complement proceeds. The RCA process can have advantages over such as techniques PCR in certain circumstances as the product generated can be very long strands of repeated linear copies of the target sequence. This is advantageous for uses such as *in situ* detection were the nature of the amplification product prevents its ready dissolution away from its site of origin. However, routine amplification and detection can be made more complex due to the large number of differently sized amplification products it generates.

Another isothermal amplification mechanism is LAMP (Loop mediated amplification). In this technique special linear primers are used which upon

polymerisation against a suitable template generate loop structures which, once copied, can be opened up by the same primer and a strand displacing DNA polymerase, to allow further looped sequences to be generated. Since these loop structures are present at both ends of the molecule the reaction is autocatalytic in isothermal conditions, which, while can be advantageous in certain circumstances, removes a degree of control. Also, the products of the amplification are DNA fragments of a range of sizes containing concatomeric strings of copies of the amplified target sequence.

The invention of Uhlen and Petersson (WO9323563) involves the use of a ‘loop generating primer’ and a linear primer which is, or can be, bound to a solid phase during or after a PCR reaction. However, the PCR reaction envisaged is a standard reaction without the use of a strand displacing DNA polymerase, which does not seek to generate increased levels of amplification from the use of such and further does not extract any information about the nature of the reaction from the data generated. The use of the loop generating primer of the invention of Uhlen and Petersson is to allow the product of the PCR reaction to be processed further, having been immobilised onto a solid phase, by performing such as a sequencing reaction on the immobilised material.

Another invention involving loop structures is the ‘Terminal Repeat Amplification’ method of Malek and Sooknanan (US5665545). This is a complex amplification mechanism involving RNA and DNA products and two primers, one of which has a loop structure. In this case the loop structure is important to the amplification process, however, the primers sequence contains the entire loop as opposed to generating it upon extension against its target. It is also an isothermal process, which does not seek to generate increased levels of amplification of a thermocycled amplification from the use of a loop generating primer and a linear primer and further does not extract any information about the nature of the reaction from the data generated and in particular from the rate of that reaction.

When comparing isothermal techniques with ‘cycled’ amplifications such as PCR, a particular advantage of the latter is that each ‘cycle’ of the process acts as its own individual extension reaction and usually sufficient time is allowed for

essentially all primers that are hybridised to target strands to be fully extended. In consequence the reaction naturally 'stops' when all primers are fully extended before the next round of denaturation reveals more primer binding sites for the reaction to reinitiate. In this way small fluctuations in chemical reaction rate between individual tubes are compensated as each reaction is run to completion before the next cycle is initiated. This allows a significance to be attached to the cycle number over which a reaction is performed. For example, PCR reactions are performed on two separate samples, A and B, under the same reaction conditions in which a detectable signal is generated in the presence of a certain number of amplified fragments. If A gives detectable signal after 15 PCR rounds while B gives a signal after 20 rounds, sample A could be inferred to have contained a larger number of template fragments at the start of the reaction since it required less amplification to reach detectable signal levels.

In many isothermal reactions the regeneration of primer binding sites is autocatalytic (it is a consequence of the amplification reaction itself) and once initiated it cannot be stopped and restarted at defined steps in the amplification process in the way that PCR can. A disadvantage of this autocatalysis is that the overall rate of amplification may be dependent on subtle differences between the chemical ingredients of individual reaction tubes. Such differences may arise from differences in the efficiency of cleaning of the DNA sample in each tube for instance, or from some other variable factor.

A second benefit of having control over the cycling of the reaction is that some measure of the efficiency of the reaction is possible. A number of techniques have been developed for homologous real-time monitoring of 'PCR–type' amplification reactions. Generally these involve the use of different combinations of primers tagged with fluorescent moieties and/or 'quencher' moieties that interact during the reaction in a way which conveys information about the amount of product formed and is measurable from the outside without interrupting the reaction. Using these techniques to follow the generation of product during a reaction it should be possible to measure the rate at which product is formed.

The general formula for estimating the number of copies of the target sequence that are present in a thermocycled amplification such as PCR after n cycles is to use an exponential function $f(n) = X^n$ where n (the exponent) refers to the number of cycles of PCR undertaken and X (the base) indicates that the number of copies of the target sequence after each cycle of the reaction per sequence before the cycle. Thus, in the case of an idealised PCR reaction, with all reagents in excess etc. a maximal base value of 2 may be achieved. This would indicate a doubling of the number of copies per cycle.

PCR is advantageous over some of the isothermal amplification methods in that it is relatively simple requiring the use of only two synthetic oligonucleotide primers and a DNA polymerase. Several isothermal amplification techniques suffer the disadvantage that they require the concerted action of several enzymes (for example SDA requires a restriction enzyme and a DNA polymerase) and often more than two oligonucleotide primers (four in the case of SDA). Other reagents may also be required, and these may include such as modified nucleotide bases for incorporation into growing DNA strands to protect them from nuclease attack (as in SDA).

A particular disadvantage of the PCR technique is that it can be prone to the generation of false positive results as a consequence of mispriming events and the generation of false bands and so-called 'primer dimers'. These aberrant amplification products are the result of incorrect copying of a template at an early stage in the reaction. Primer dimers result from the formation of a hybrid molecule having at its ends the sequences of one primer and the complement of the sequence of the other, and hence a template for PCR amplification. Other forms of mispriming occur when the stringency of the reaction is not optimal and secondary binding (and extension) sites for one or both primers occur in positions which allow a product to be generated that can act as a substrate for further amplification. This generally results in a product band of different size and sequence to that expected. Since all these aberrant products possess sites for the authentic primers at their ends they are then amplified in exactly the same manner and at the same rate as the authentic target fragments.

Details of the Invention

The aforementioned disadvantages of current amplification methods are addressed in the method of the invention and a method of nucleic acid amplification is presented which is advantageous over the current state of the art.

According to a first aspect of the present invention there is provided a method of amplifying a nucleic acid sequences comprising the steps of

(i) providing a reaction mixture comprised of

(a) a polymerase enzyme system including a strand displacing polymerase,

(b) a target nucleic acid strand incorporating the sequence to be amplified,

(c) a first primer having a 3' region capable of hybridising to the target nucleic acid strand and copying said sequence as a template to form a first primer extension product, and a 5' region capable of self-hybridising to said first primer extension product to form a first single stranded loop structure, and

(d) a second primer capable of being hybridised to the target strand to copy the target strand back towards the loop to form a second primer extension product which is capable of self-hybridisation to form a second single stranded loop structure to which said first primer is capable of being hybridised and then extended; and

(ii) effecting repeated cycles of hybridisation, polymerisation and denaturation to effect amplification of said sequence.

According to a second aspect of the present invention there is provided a method of amplifying a nucleic acid sequences comprising the steps of

(i) providing a reaction mixture comprised of

(a) a polymerase enzyme system including a strand displacing polymerase,

(b) a target nucleic acid strand which has or is capable of forming a single stranded loop and which incorporates the sequence to be amplified,

(c) a first primer, and

(d) a second primer capable of being hybridised to the first primer extension product to copy the extension product back towards the loop to form a second primer extension product which is capable of self-hybridisation to form a second single stranded loop structure to which said first primer is capable of being hybridised and then extended; and

(ii) effecting repeated cycles of hybridisation, polymerisation and denaturation to effect amplification of said sequence.

In the method of the invention, the hybridisation and polymerisation steps may (but not necessarily) be conducted at the same temperature. The denaturation step will be conducted at a higher temperature than either the hybridisation and polymerisation steps.

The manner in which the method of the invention effects amplification of a nucleic acid strand is exemplified more fully below.

However, in summary, the method of the invention proceeds (subsequent to hybridisation) with strand displacement polymerisation to a point where double stranded molecules are formed and need to be denatured before the reaction can proceed further. Subsequent to denaturation, hybridisation and polymerisation again continues to the point where double stranded molecules are formed and need to be denatured before the reaction can proceed further. Thus, in the method of the invention, a target nucleic acid strand is amplified by a method which combines the strand separation technique (such as employed in PCR) with a separation independent strand displacement method. The method of the invention maintains the advantageous

property of PCR that the reaction will naturally 'stop' after a time in the absence of a strand separating mechanism and can hence be put under cyclic control.

The technique has the further advantage that a small and predictable series of amplification reactions can occur within each cycle to achieve overall rates of amplification higher than achievable by PCR alone. When using the exponential function $f(n) = X^n$ outlined above, rates of amplification may be achieved wherein base X is greater than achievable by a PCR-type amplification under the same reaction conditions and preferably base X is greater than 2 and more preferably base X may be 2.5 – 3. One advantage of this enhanced amplification rate is that reaction products may be accumulate faster than former methods (in terms of time taken and/or the number of cycles performed). A further advantage is that the rate of accumulation of 'true' reaction products is higher than the rate at which common mispriming products will accumulate and so will come to dominate a reaction in which such products are present.

In this way greater sequence specificity can be inferred from a positive result of the method of the invention than for the conventional amplification technique outlined. Several fluorophore/quencher techniques have been developed to overcome the lack of specificity of the types of homogenous detection discussed (e.g. TaqMan, Scorpion primers, Sunrise primers etc (all trade names)) and these techniques may also find utility in the method of the invention.

An advantage of the invention is that the number of different products of the amplification that occurs within each round of the process is limited and are the same throughout the process. This is in contrast to such as the Rolling Circle or LAMP techniques outlined above in which an ever more complex mixture of fragments is produced. The effect of this is that a standard reaction time can be determined which might then apply to all cycles of the reaction. A second advantage is that the products of the method of the invention should be easily interpretable from such as agarose gel electrophoresis.

A specific disclosure of the method of the invention is that the rate of product formation be monitored during the progress of the reaction and that this be used to

determine whether the reaction is a 'true' amplification of the desired sequence or a 'false' positive reaction. In the method of the invention the 'true' reaction should be seen to proceed at rates greater than achievable by conventional PCR reactions under the conditions used and preferably should proceed at greater than a doubling of product with each cycle of the reaction (base $X > 2$). In practice the detection of reaction product occurs fairly late in the reaction when reagent utilisation and enzyme inactivation may make hypothetical rates unattainable, however in like conditions the method of the invention should allow product generation to occur at a rate detectably faster than that for 'conventional' PCR or for false positive reaction products.

The ability to distinguish 'true' from 'false' reactions on the basis of rate of product formation in a step-wise (usually thermocycled) manner has beneficial consequences. A number of homogenous 'real-time' detection systems are less favoured in the art because the signal they produce is not considered sufficiently sequence specific. This as because a mis-priming event which generates an amplifiable product will generate signal at the same rate as a 'genuine' reaction product would. Thus it is impossible, without further investigation, to distinguish false reactions from the reaction obtained from (albeit low levels of) genuine product. The heightened amplification rates obtained by a 'true' reaction of the method of the invention will firstly generate far more product in a given number of cycles than a 'conventional' reaction and therefore will reach detectable levels earlier, and secondly once product formation is detectable from cycle to cycle it should achieve amplification rates ($>2^n$) which are not possible by 'false' reactions and so the rate of the reaction is diagnostic of a 'true' reaction.

At its simplest the technique requires only two synthetic oligonucleotides and a strand displacing DNA polymerase. Typical strand displacing DNA polymerase enzymes which might be used in the method of the invention include Vent, Vent(exo-), Deep Vent, Deep Vent(exo-) and 9° North (all trade marks, New England Biolabs). Other thermostable DNA polymerases are known in the art and the ability of some of these enzymes to perform strand displacing DNA polymerisation is not reported. Such enzymes that are able to carry out strand displacing DNA polymerisation would also find utility in the method of the invention.

The target nucleotide strand for the method of the invention is wholly or partially double stranded possessing a hairpin loop at one end. At least two oligonucleotide primers are used. The first primer will be homologous along a part or whole of its length to initiate DNA polymerisation upon hybridisation to the target. The second will be wholly or largely complementary at its 3' end to the free nucleotides of the loop portion of the hairpin loop or to the complementary sequence to said free nucleotides. The 5' end of the second primer will be largely or wholly homologousto a sequence of nucleotides 'downstream' (i.e. in the 3' direction) of the free nucleotides of the loop portion of the hairpin loop or 'upstream' (i.e. in the 5' direction) of the complementary sequence to said free nucleotides. Both primers of the method of the invention are linear in nature i.e. they are not designed to assume a looped secondary structure in the absence of a target template.

It is known in the art that 'looped' primers may be used in a conventional PCR reaction and an example of this would be the use of primers carrying a 5' hairpin loop carrying a fluorophore moiety and a quencher moiety from a fluorescence resonance energy transfer (FRET) pair such that, in conditions where the hairpin loop is intact the quencher absorbs fluorescence energy from the excited fluorophore such that said energy is not emitted as fluorescence. Upon incorporation into a double stranded PCR molecule the fluorophore and quencher are physically separated and no such quenching can occur and as a result a fluorescent signal is generated.

The use of such 'looped' primers in a conventional PCR reaction does not anticipate two important teachings of the method of the invention.

The first teaching of the method of the invention is that by using a loop generating primer and a conventional primer in a thermocycled amplification reaction with a strand displacing nucleic acid polymerase a higher level of amplification may be attainable than that attained in each cycle of an amplification reaction such as PCR. The reason for using a looped primer for the reactions outlined above is not to achieve an increased level of amplification (as in the method of the invention) but rather to detect amplification by fluorescence generation. Because of the nature of the molecules used some enhancement of the level of amplification may be obtained in a

conventional PCR reaction, but, this would occur only where a strand displacing DNA polymerase were used (and it is not conventional to use such an enzyme in PCR) and the level of enhancement would be less than that described for the method of the invention. This would be because the looped primer is not designed to hybridise at its 3' end to the single stranded portion of the hairpin loop target (as would be required to open up the target to copy downstream sequences). Any enhancement to the amplification rate conferred by the use of one conventional primer and one looped primer would arise because the looped primer might hybridise to copies of the target before it adopted a hairpin loop formation. This could then copy the target from its hybridised position. Subsequently, this copy could be displaced by extension from the conventional primer hybridised upstream, but this would only occur in the presence of a strand displacing nucleic acid polymerase.

The second teaching is that the level of amplification of the method of the invention is higher where the authentic reaction occurs than were fortuitous mis-priming events occur. To achieve the increased amplifications rates of the method of the invention several sequence specific events must occur. The template must be of a hairpin loop structure, such that it may be 'super' amplified by the concerted actions of two linear primers, and the primers must act as described , i.e. one as a conventional primer and one as a loop generating primer. In the case where a looped primer and a conventional primer were used any mis-priming event which resulted in an hybrid molecule amplifiable by the looped primer alone or in concert with the regular primer would generate product at the same rate as the authentic target. This is not the case in the method of the invention.

A particularly advantageous embodiment of the method of the invention involves the use of one 'conventional' oligonucleotide primer and one 'loop generating' primer that is structured such that at its 3' end it is sufficiently complementary to the target sequence to hybridise to it and to act as a point of initiation of DNA polymerisation and which has at least one further region 5' to this which is sufficiently complementary to a portion of the DNA strand target that is created by polymerisation from the aforementioned 3' hybridising region against the target template to hybridise to it and to act as a point of initiation of DNA polymerisation.

In this manner the loop generating primer will naturally generate the hairpin loop target of the invention when the 3' end is extended against the target and the strands are then separated and allowed to self hybridise.

The invention would find obvious application in the study of mutations and polymorphisms such as single nucleotide polymorphisms (SNPs) but may find particular application in haplotyping studies. Haplotyping seeks to determine the strand association of multiple mutations. For instance two SNP loci , X and Y, are characterised and it is known that common polymorphisms generate C or A at position X and T or C at position Y. A typical SNP analysis of , for instance a patient sample, may determine that the subject is heterozygotic for both SNPs (i.e. carries both types of SNP at both sites, that is they give signals indicative of both C and A at position X and of T and C at Y). A more complex study would be needed to determine the association on each diploid strand as two combinations are possible (CxCy/AxTy and CxTy/AxCy). Since the method of the invention requires three levels of sequence specificity and two of these are associated with one primer, it is possible to design the loop generating primer to have regions specific to both SNPs such that only the true haploid strand complementary to this sequence at both sites would be able to make a target template of the method of the invention and thus by amplified at the higher rate described above. A common second primer may then be used as the second primer of the method of the invention.

According to a third aspect of the invention there is provided a means of amplifying a target nucleic acid sequence which forms or is capable of forming a hairpin loop structure comprising a single strand of nucleic acid a portion of which can hybridise to another portion of same to produce a single stranded loop structure connected to a partly or wholly double stranded base sequence said amplification means comprising;

i) treating the target strand with at least one first oligonucleotide primer which has at its 3' end a region which is capable of hybridising to the free nucleotides of said loop structure or to the complement of such structure and

of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents and has at its 5' end a region capable of hybridising to the product of such polymerisation and at least one second oligonucleotide primer capable of hybridising to a site on the nucleotide strand upstream of the site of hybridisation of said first primer and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents under conditions such that said hybridisation can occur and such that an extension product will be produced from hybridised primers complementary to their templates and further using an agent for said polymerisation which is capable of displacing downstream hybridised nucleic acid sequences during extension,

ii) separating the double stranded regions of (i) to render them single stranded, and

iii) repeating the steps of (i) and (ii)

According to a fourth aspect of the invention there is provided a means of amplifying a target nucleic acid strand comprising;

i) treating the target strand with at least one first oligonucleotide primer which has at its 3' end a region which is capable of hybridising to the target and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents and has at its 5' end a region capable of hybridising to the product of such polymerisation and at least one second oligonucleotide primer capable of hybridising to a site on the nucleotide strand complementary to the target strand or to the product of extension of the first primer and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents under conditions such that said hybridisation can occur and such that an extension product will be produced from hybridised primers complementary to their templates and further using an agent for said polymerisation which is capable of displacing downstream hybridised nucleic acid sequences during extension,

ii) separating the double stranded regions of (i) to render them single stranded, and

iii) repeating the steps of (i) and (ii)

According to a fifth aspect of the invention there is provided a means of amplifying a target nucleic acid sequence which forms or is capable of forming a hairpin loop structure comprising a single strand of nucleic acid a portion of which can hybridise to another portion of same to produce a single stranded loop structure connected to a partly or wholly double stranded base sequence and detecting the product of said amplification during the reaction, said amplification means comprising;

i) treating the target strand with at least one first oligonucleotide primer which has at its 3' end a region which is capable of hybridising to the free nucleotides of said loop structure or to the complement of such structure and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents and has at its 5' end a region capable of hybridising to the product of such polymerisation and at least one second oligonucleotide primer which carries a moiety that is fluorescent when illuminated with light of suitable excitation wavelength and has at its 3' end a region which is capable of hybridising to a site on the nucleotide strand upstream of the site of hybridisation of said first primer and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents and has at its 5' end a region capable of hybridising to a third oligonucleotide which carries a moiety that interacts which said fluorescent moiety to inhibit its fluorescence when the second and third oligonucleotide primers are hybridised under conditions such that said hybridisation can occur and such that an extension product will be produced from hybridised primers complementary to their templates and further using an agent for said polymerisation which is capable of displacing downstream hybridised nucleic acid sequences during extension,

ii) separating the double stranded regions of (i) to render them single stranded,

iii) repeating the steps of (i) and (ii), and

iv) monitoring the rate of the reaction by detecting the level of fluorescence emitted from the reaction mix when suitably illuminated.

According to a sixth aspect of the invention there is provided a means of amplifying a target nucleic acid sequence which forms or is capable of forming a hairpin loop structure comprising a single strand of nucleic acid a portion of which can hybridise to another portion of same to produce a single stranded loop structure

connected to a partly or wholly double stranded base sequence and detecting the product of said amplification during the reaction, said amplification means comprising;

treating the target strand with at least one first oligonucleotide primer which has at its 3' end a region which is capable of hybridising to the free nucleotides of said loop structure or to the complement of such structure and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents and has at its 5' end a region capable of hybridising to the product of such polymerisation and at least one second oligonucleotide primer which is capable of hybridising to a site on the nucleotide strand upstream of the site of hybridisation of said first primer and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents including a reagent that changes its fluorescence characteristics upon interaction with double stranded DNA under conditions such that said hybridisation can occur and such that an extension product will be produced from hybridised primers complementary to their templates and further using an agent for said polymerisation which is capable of displacing downstream hybridised nucleic acid sequences during extension,

i) separating the double stranded regions of (i) to render them single stranded,
ii) repeating the steps of (i) and (ii), and
iii) monitoring the rate of the reaction by detecting the level of fluorescence emitted from the reaction mix when suitably illuminated.

According to a seventh aspect of the invention there is provided a means of amplifying a target nucleic acid sequence which forms or is capable of forming a hairpin loop structure comprising a single strand of nucleic acid a portion of which can hybridise to another portion of same to produce a single stranded loop structure connected to a partly or wholly double stranded base sequence and detecting the product of said amplification during the reaction, said amplification means comprising;

i) treating the target strand with at least one first oligonucleotide primer which has at its 3' end a region which is capable of hybridising to the free nucleotides of said loop structure or to the complement of such structure and of initiating DNA polymerisation from this hybridisation site in the presence

of suitable reagents and has at its 5' end a region capable of hybridising to the product of such polymerisation and at least one second oligonucleotide primer which is capable of hybridising to a site on the nucleotide strand upstream of the site of hybridisation of said first primer and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents including a reagent that interacts with double stranded DNA in such a way as to alter the fluorescence characteristics of the whole when the reagent and said double stranded DNA are brought into close proximity as by such as intercalation of the reagent into the double stranded DNA under conditions such that said hybridisation can occur and such that an extension product will be produced from hybridised primers complementary to their templates and further using an agent for said polymerisation which is capable of displacing downstream hybridised nucleic acid sequences during extension,

ii) separating the double stranded regions of (i) to render them single stranded,

iii) repeating the steps of (i) and (ii), and

iv) monitoring the rate of the reaction by detecting the level of fluorescence emitted from the reaction mix when suitably illuminated.

Typical reagents used in the seventh aspect of the invention would include intercalating fluorescent dyes such as Ethidium Bromide or PicoGreen (Trade name, Molecular Probes).

The methods of the third to sixth aspects of the invention may be conducted without recause to opening the vessel in which the amplification is occurring for the purpose of detecting the amplification product formed during the reaction.

Examples

The following non-limiting examples illustrate the method of the invention with reference to the attached figures in which;

Figure 1 shows how a hairpin loop target nucleic acid of the method of the invention may be formed by extension of a suitably configured linear primer against a linear target nucleic acid;

Figure 2 shows how a hairpin loop target nucleotide may be amplified by the method of the invention;

Figure 3 shows how one of the intermediate double stranded amplification products is further amplified following strand separation by the method of the invention;

Figure 4 shows how another intermediate double stranded amplification product may be further amplified following strand separation by the method of the invention;

Figure 5 illustrates a comparative nucleic acid amplification process employing luminescence to follow progress of an amplification reaction;

Figure 6a and 6b illustrates an embodiment of the method of the invention employing luminescence to follow progress of the amplification reaction; and

Figure 7 illustrates the method of the invention for detection of the sequence association of two polymorphic sites.

The mechanism by which the method of the invention effects amplification of a nucleic acid is described fully below with reference to Fig 1-4. However, in summary, Fig 1 may be regarded as the starting point for a method of the first aspect of the invention which then proceeds through the steps of Figs 2-4. Fig 2 may itself be regarded as the starting point for a method in accordance with the second aspect of the invention which then proceeds through the steps of Figs 3 and 4. By way of further summary, the procedure of Fig 2 leads to the production of double stranded molecules 15 and 17 such that the amplification procedure "stops" until these molecules are denatured. Subsequent to denaturation and then hybridisation with other moieties in the reaction mixture, the double stranded molecules 15 and 17 are amplified by the procedures illustrated in Figs 3 and 4 respectively. It will be seen from Fig 3 that the double stranded molecule 15 is converted (by the mechanism illustrated in Fig 3) to a double stranded molecule 17. It will be seen from Fig 4 that a

double stranded molecule 17 is converted into a double stranded molecule 15 and a further copy of soluble stranded molecule 17. Thus with each round of amplification there are produced (as amplification products) the double stranded molecules 15 and 17 in a ratio of two molecules 17 for each molecule 15.

Referring to fig 1, a hairpin loop target (4) is created by the polymerisation of a loop generating primer (2) against a linear template (1). The example in Figure 1 shows a linear nucleic acid strand (1) possessing the regions of sequence (5' to 3') of C, X, B, A' where C is the sequence of the 3' end of a 'conventional' primer, X is a region to be amplified and A' and B are regions that will interact with the loop generating primer (2) of sequence having regions (5' to 3') of B also shown. The linear nucleic acid strand (1) and loop generating primer (2) are hybridised together at the complementary A/A' region (see step (i)). The loop generating primer is then extended by the action of a nucleic acid polymerase to generate a complementary copy (3) of the target strand (1) from the primer (2) whose sequence has regions B, A, B', X' C' (ii). When these two strands (1) and (3) are separated and allowed to self anneal (iii) the copy of the linear strand from the loop generating primer can adopt the hairpin loop structure (4).
Referring now to Fig 2, the target hairpin loop (4) is hybridised with an oligonucleotide primer (5) of sequence C (ii). Primer (5) is then extended against the target hairpin loop (4) by the strand displacing nucleic acid polymerase (iii) to give an extended copy (6) (of primer (5)) whose sequence has regions C, X, B, A', B'. Separation and self hybridisation of the products (iv) will regenerate the target hairpin loop (4) and a second hairpin loop structure (7). Since the regenerated hairpin loop (4) is the identical strand to that in step (i) it will act as template for extension of a copy of oligonucleotide primer C (5) to make an extended copy (6) of said primer at which point it will 'stop' until the strands are separated at the next cycle of the procedure.

In the presence of a suitable nucleic acid polymerase and conditions the second hairpin loop (7) will undergo the following reactions. For simplicity's sake each reaction will be shown to occur temporally separately from any other reaction, in step-wise fashion. This need not necessarily be the case in practice when more than one reaction might occur at the same time. The free 3' end (8) of the hairpin loop (7) will be extended by the polymerase to 'fill in' the single stranded region of the hairpin

(v) to generate a hairpin loop with a fully double stranded base (9) and a single stranded loop (10). This single stranded loop (10) is complementary to the 3' end of the loop generating primer (11) whose sequence contains regions BA (11) and so this primer will hybridise (vi) to the area of complementarity in the loop (12) and from there will be extended by the strand displacing polymerase (vii) to generate a partially single stranded linear molecule (13).

The single stranded region of the molecule (13) contains a region C' to which primer (14) will bind (viii). The primer (14) will be extended by the strand displacing polymerase (ix) to generate one completely double stranded nucleic acid (15) and one partially single stranded hairpin loop structure (16). The double stranded molecule (15) will take no further part in the amplification reaction until the strands are again separated. The region C' of single strand in the base of the hairpin loop (16) is complementary in sequence to the primer (14) containing sequence C at its 3' end and so said primer will bind at this region of complementarity (x). The primer (14) will then be extended from the hybridisation site (xi) to render the molecule completely double stranded and the reaction will 'stop' until the strands are separated again.

While the initial reactions in the method maybe somewhat confusing it is clear that after a short series of reactions, in which extra copies of the target sequence X are made, the reactions come to a natural end and will not proceed further until the double stranded molecules are separated.

Two particular types of double stranded product will come to dominate the amplification reaction as it proceeds. This are a long double stranded form (15) which contains the entire sequence of the target in double strand form, and a second shorter version (17) which contains a single copy of the target bounded on one side by a second copy of the loop generating sequence.

Figure 3 shows the steps in the amplification of the longer double strand (15) in subsequent rounds of amplification. (amplification of the shorter double stranded molecule 17 is described below with reference to Fig 4).

The strands of the longer double strand product (i) are separated to generate single strands (18) and (19) and then subjected to conditions which will promote hybridisation of complementary regions (ii).

In the example shown the strands are shown to hybridise to the primers of the method of the invention. In reality a competition will occur for hybridisation sites between the primers and the other complementary regions on the molecule itself or between complementary sites on other product molecules. In the case of the first single strand (18) self hybridisation may mask the site C' thus preventing hybridisation of the primer containing region C. This strand will then not amplify until a subsequent separation of the hybridised regions at the start of the next cycle of the amplification. In the case of the second single strand (19) such self hybridisation will not have such an effect upon subsequent hybridisation as the self hybridisation of the second single strand (19) will leave a single strand loop to which the loop generating primer may hybridise. Subsequent polymerisation from this primer will unravel the hybridised regions by displacement which will allow the full amplification reaction to occur.

In circumstance in which primer (14) hybridises to the first single strand (18) and is polymerised against it (iii), the molecule will be rendered double stranded (and hence indistinguishable from the original double stranded molecule (15)) and will then take no further part in this round of amplification. The initial products of amplification of the second single strand (19) are relatively independent of the mechanism of their performance, as outlined above, and the products are a copy of the original double stranded molecule (15) and a shorter partially single stranded hairpin loop structure (16) which has at its 3' end the sequence C' to which the primer (14) carrying region C may hybridise (iv) and be extended (v) to render the molecule double stranded (17). Thus the products of a single amplification round of the longer double stranded molecule (15) are two identical molecules (15) and one shorter double stranded molecule (17).

Figure 4 shows the steps in the amplification of the shorter double strand (17) in subsequent rounds of amplification. The strands of the shorter double strand product (17)(i) are separated and then placed in conditions which will promote

hybridisation of complementary regions (ii) generating the partially single stranded hairpin loops (16) and (7).

The region of single strand in the base of the hairpin loop (16) is complementary in sequence to the primer (14) containing sequence C at its 3' end and so said primer (14) will bind at this region of complementarity (ii). The primer (14) will then be extended from the hybridisation site (iii) to produce the completely double stranded molecule (17) and the reaction will 'stop' until the strands are separated again.

In the presence of a suitable nucleic acid polymerase and conditions the second hairpin loop (7) will undergo the following reactions. For simplicities sake each reaction will be shown to occur temporally separately from any other reaction, in step-wise fashion. This need not necessarily be the case in practice when more than one reaction might occur at the same time. The free 3' end (8) of the hairpin loop (7) will be extended by the polymerase to 'fill in' the single stranded region of the hairpin (iii) to generate a hairpin loop with a fully double stranded base (9) and a single stranded loop (10). This single stranded loop (10) is complementary to the 3' end of the loop generating primer (11) whose sequence contains regions BA and so this primer (11) will hybridise (iv) to the area of complementarity in the loop (as represented by reference numeral (12)) and from there will be extended by the strand displacing polymerase (v) to generate a partially single stranded linear molecule (13).

The single stranded region of the molecule (13) contains a region complementary to the primer (14) containing sequence C at its 3' end and so this primer will bind at this region of complementarity (vi)and be extended by the strand displacing polymerase (vii) to generate one completely double stranded nucleic acid (15) and one partially single stranded hairpin loop structure (16). The double stranded molecule (15) will take no further part in the amplification reaction until the strands are again separated. The region of single strand in the base of the hairpin loop (16) is complementary in sequence to the primer (14) containing sequence C at its 3' end and so this primer will bind at this region of complementarity (viii). The primer (14) will then be extended from the hybridisation site (ix) to render the molecule completely double stranded and the reaction will 'stop' until the strands are separated again.

Thus the products of a single amplification round of the shorter double stranded molecule (17) are two identical molecules (17) and one longer double stranded product (15).

Figure 5 illustrates the 'real time' detection technology in a conventional PCR amplification reaction with the method of the invention and indicates how this is advantageous of conventional amplification means.

An amplification mix is prepared containing one 'conventional' primer (18), a second primer (19) and a third synthetic oligonucleotide (20) which is present in molar equivalence or excess of (19). Primer (19) has two regions in its sequence, at its 3' end it has a target specific sequence (21) in the same way as a 'conventional' primer would. At its 5' end it possesses a sequence (22) complementary to the sequence of synthetic oligonucleotide (20). Furthermore, primer (19) has at its 5' terminus a fluorescent moiety (23) while oligonucleotide (20) has at its 3' terminus a quencher (24) for said fluorophore (23) which is such that, under conditions in which oligonucleotide (20) becomes hybridised to primer (19) at their complementary sequences, it absorbs the energy generated when the fluorophore absorbs light, and hence no fluorescent signal is generated. A template (25) is added and a 'conventional' PCR reaction is performed during which the reaction is probed by illuminating the samples with light of the excitation wavelength of the fluorophore and recording the fluorescence signal produced. The three synthetic molecules will separate during the denaturation step, generating a high fluorescence signal (i), but during the subsequent hybridisation step primer (19) and oligonucleotide (20) will hybridise at their regions of complementarity and oligonucleotide (20) will 'quench' the signal from primer (19) and a weak signal will be generated. As the amplification proceeds primer (19) will become incorporated into copies of the target such that extension from primer (18) on target copies containing primer (19) (ii) will displace oligonucleotide (20) into solution (or degrade it) such that it no longer blocks signal from the fluorophore on primer (19) and a stronger signal is produced (iii). The strength of this signal will indicate the amount of product in the reaction and the rate of increase of signal at the end of consecutive extension steps will reflect the rate of product formation.

Consider the same reaction conducted in the absence of suitable template. A mispriming event occurring early in the reaction (iv), creates a molecule (26) having at its ends the sequence of primer (18), and a sequence complementary to primer (19) (or vice versa) and is amplified (v) at a rate matching that of genuine product . The detection system will detect a different level of product (if the number of misprimed sequences originally generated was less than that expected from 'genuine' product), but it cannot distinguish a difference in reaction rates between the two, 'false' and 'true' products. Thus, in the system outlined the detection of a signal is not sufficiently sequence dependent to differentiate artefacts of the reaction (misprimes etc.) from low levels of 'genuine' product.

Figure 6a shows a similar reaction conducted as in the method of the invention. An amplification mix is prepared containing (i) a 'loop generating' primer (27), whose sequence has regions 5' – BA – 3', a second a primer (28), whose sequence has regions 5' – DC – 3', and a third synthetic oligonucleotide (29), whose sequence is D' and which is present in molar equivalence or excess of primer (28). As before Primer B has two regions in its sequence, it has a target specific 3' end and a 5' end complementary to C allowing B and C the potential to form a fluorophore/quencher pair and this interaction monitored as above. In the presence of target (30) three sequence specific events need to occur to generate the 'super exponential amplification' of the invention. Firstly one or other primer (A or B) must recognise its site (ii) and be extended against it (iii). Second, once this extension product is separated from its template (iv) it must be recognised and extended by the opposite primer (v) . Lastly, the loop forming portion of primer A must recognise its complement (vi) within this extension product to allow loop formation. Incorporation of fluorescently tagged primers into such amplification products will lead to displacement of their hybridised 'quencher' oligonucleotides (vii) and a fluorescent signal will be generated. If these three sequence specific events occur exponential amplification ($>2^n$) will generate copies of the target and this heightened rate of product generation should be detectable by following the generation of this fluorescent signal..

In the absence of target template similar mispriming events may occur to those explained above (see Fig 6b) and these will be amplified, however, the rates amplification of these misprimed products will be of the 'ordinary' (2^n) level. As a consequence these 'false' products will appear at the same approximate cycle number as in the previous 'conventional' amplification, while 'true' products should appear much earlier. Also once the 'false' products reach detectable levels their rate of amplification between cycles will be detectably lower (2^n or below) than for 'true' product.

Figure 7 illustrates an embodiment of the method of the invention for detection of the sequence association of two polymorphic sites and might be used in such as a 'haplotyping' study. Consider a DNA sample containing copies of a target region from two chromosomes which have polymorphisms at positions A and B such that one chromosome has the haplotype A'B while the other has haplotype a'b. Four reactions are prepared using four different loop generating primers covering all combinations of A and B polymorphism, together with a single common upstream primer C (i). After hybridising to their complementary 'A' sites and being extended (ii) the extension products are then separated and allowed to self anneal (iii). It can be seen that only those loop generating primers matching one of the haplotypes in the sample generate a hairpin loop target and only these would therefore give the heightened rates of amplification of the method of the invention when amplified with common primer C.

Figures 8 and 9 illustrate an embodiment of the method of the invention for detection of the sequence polymorphism in a sample in which any of two or more sequence variants may be present in the sample. Consider a DNA sample containing two copies of a chromosome carrying a target region of sequence ABC'. Within this sequence is a polymorphism such that either chromosome may carry the allele X or Y with region B. A reaction is prepared using a loop generating primer containing at its 3' end sequence A and at its 5' end sequence B containing the X allele of the polymorphism, together with a single upstream primer C (i).

Figure 8 illustrates how, in the presence of the matching allelic target a reaction in accordance with the invention is initiated. After hybridising to their

complementary 'A' sites (ii) and being extended (iii) the extension products are then separated and complementary strands then allowed to anneal (iv). Following further extension from the annealed primer C (v), separation and hybridisation (vi) the fully complementary partially double stranded hairpin is formed. This can be extended by a suitable nucleic acid polymerase enzyme (vii) and forms a product that can be amplified by the method of the invention at per cycle rates higher than PCR.

Figure 9 illustrates that, in the presence of target carrying the mismatch allele Y and following similar steps of repeated hybridisation, extension and separation, the partial double stranded hairpin formed (vi) is not perfectly complementary and not a suitable substrate for accelerated amplification by the method of the invention. This substate can still be amplified by a mechanism similar to PCR, in the presence of a suitable polymerase, and will lead to product formation at a rate less than or equal to that of PCR (X <=2). Thus the perfectly matched and mismatched targets may be differentiated by measuring the rate of product accumulation from these reactions.

CLAIMS

1. A method of amplifying a nucleic acid sequences comprising the steps of

(i) providing a reaction mixture comprised of

(a) a polymerase enzyme system including a strand displacing polymerase,

(b) a target nucleic acid strand incorporating the sequence to be amplified,

(c) a first primer having a 3' region capable of hybridising to the target nucleic acid strand and copying said sequence as a template to form a first primer extension product, and a 5' region capable of self-hybridising to said first primer extension product to form a first single stranded loop structure, and

(d) a second primer capable of being hybridised to the target strand to copy the target strand back towards the loop to form a second primer extension product which is capable of self-hybridisation to form a second single stranded loop structure to which said first primer is capable of being hybridised and then extended; and

(ii) effecting repeated cycles of hybridisation, polymerisation and denaturation to effect amplification of said sequence.

2. A method of amplifying a nucleic acid sequences comprising the steps of

(i) providing a reaction mixture comprised of

(a) a polymerase enzyme system including a strand displacing polymerase,

(b) a target nucleic acid strand which has or is capable of forming a single stranded loop and which incorporates the sequence to be amplified,

(c) a first primer, and

(d) a second primer capable of being hybridised to the first primer extension product to copy the extension product back towards the loop to form a

second primer extension product which is capable of self-hybridisation to form a second single stranded loop structure to which said first primer is capable of being hybridised and then extended; and

(ii) effecting repeated cycles of hybridisation, polymerisation and denaturation to effect amplification of said sequence.

3. A method as claimed in claim 1 or 2 wherein

the reaction mixture incorporates a third oligonucleotide primer capable of hybridising to the 5' region of the second oligonucleotide primer; and

one of the second primer and third primer carries a fluorescent moiety and the other carries a fluorescence modifier which modifies fluorescence from said fluorescent moiety when the third primer is hybridised to the second primer.

4. A method as claimed in claim 3 wherein the fluorescent moiety is carried by the second primer.

5. A method as claimed in claim 3 or 4 wherein the fluorescence modifier is a fluorescence quencher.

6. A method as claimed in claim 1 or 2 wherein

the reaction mixture includes a reagent that changes its fluorescence characteristics upon interaction with double stranded DNA.

7. A method as claimed in claim 6 wherein said interaction is intercalation of the reagent into the double stranded DNA.

8. A means of amplifying a target nucleic acid sequence which forms or is capable of forming a hairpin loop structure comprising a single strand of nucleic acid a portion of which can hybridise to another portion of same to produce a single

stranded loop structure connected to a partly or wholly double stranded base sequence said amplification means comprising;

(i) treating the target strand with at least one first oligonucleotide primer which has at its 3' end a region which is capable of hybridising to the free nucleotides of said loop structure or to the complement of such structure and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents and has at its 5' end a region capable of hybridising to the product of such polymerisation and at least one second oligonucleotide primer capable of hybridising to a site on the nucleotide strand upstream of the site of hybridisation of said first primer and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents under conditions such that said hybridisation can occur and such that an extension product will be produced from hybridised primers complementary to their templates and further using an agent for said polymerisation which is capable of displacing downstream hybridised nucleic acid sequences during extension.

(ii) separating the double stranded regions of (i) to render them single stranded, and

(iii) repeating the steps of (i) and (ii)

9. A means of amplifying a target nucleic acid strand comprising;

(i) treating the target strand with at least one first oligonucleotide primer which has at its 3' end a region which is capable of hybridising to the target and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents and has at its 5' end a region capable of hybridising to the product of such polymerisation and at least one second oligonucleotide primer capable of hybridising to a site on the nucleotide strand complementary to the target strand or to the product of extension of the first primer and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents under conditions such that said hybridisation can occur and such that an extension product will be produced from hybridised primers complementary to their templates and further using an agent for said

polymerisation which is capable of displacing downstream hybridised nucleic acid sequences during extension,

(ii) separating the double stranded regions of (i) to render them single stranded, and

(iii) repeating the steps of (i) and (ii)

10. A means of amplifying a target nucleic acid sequence which forms or is capable of forming a hairpin loop structure comprising a single strand of nucleic acid a portion of which can hybridise to another portion of same to produce a single stranded loop structure connected to a partly or wholly double stranded base sequence and detecting the product of said amplification during the reaction, said amplification means comprising;

(i) treating the target strand with at least one first oligonucleotide primer which has at its 3' end a region which is capable of hybridising to the free nucleotides of said loop structure or to the complement of such structure and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents and has at its 5' end a region capable of hybridising to the product of such polymerisation and at least one second oligonucleotide primer which carries a moiety that is fluorescent when illuminated with light of suitable excitation wavelength and has at its 3' end a region which is capable of hybridising to a site on the nucleotide strand upstream of the site of hybridisation of said first primer and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents and has at its 5' end a region capable of hybridising to a third oligonucleotide which carries a moiety that interacts which said fluorescent moiety to inhibit its fluorescence when the second and third oligonucleotide primers are hybridised under conditions such that said hybridisation can occur and such that an extension product will be produced from hybridised primers complementary to their templates and further using an agent for said polymerisation which is capable of displacing downstream hybridised nucleic acid sequences during extension,

(ii) separating the double stranded regions of (i) to render them single stranded, and

(iii) repeating the steps of (i) and (ii), and

(iv) monitoring the rate of the reaction by detecting the level of fluorescence emitted from the reaction mix when suitably illuminated.

11. A means of amplifying a target nucleic acid sequence which forms or is capable of forming a hairpin loop structure comprising a single strand of nucleic acid a portion of which can hybridise to another portion of same to produce a single stranded loop structure connected to a partly or wholly double stranded base sequence and detecting the product of said amplification during the reaction, said amplification means comprising;

(i) treating the target strand with at least one first oligonucleotide primer which has at its 3' end a region which is capable of hybridising to the free nucleotides of said loop structure or to the complement of such structure and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents and has at its 5' end a region capable of hybridising to the product of such polymerisation and at least one second oligonucleotide primer which carries at least one moiety that is fluorescent when illuminated with light of suitable excitation wavelength and which is capable of hybridising to a site on the nucleotide strand upstream of the site of hybridisation of said first primer and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents including a reagent that interacts with said fluorescent moiety to alter its fluorescence characteristics when the fluorescent moiety and said reagent are brought into close proximity and can further interact with double stranded DNA strands in order to adopt said close proximity when the second oligonucleotide primer is rendered double stranded under conditions such that said hybridisation can occur and such that an extension product will be produced from hybridised primers complementary to their templates and further using an agent for said polymerisation which is

capable of displacing downstream hybridised nucleic acid sequences during extension,

(ii) separating the double stranded regions of (i) to render them single stranded,

(iii) repeating the steps of (i) and (ii), and

(iv) monitoring the rate of the reaction by detecting the level of fluorescence emitted from the reaction mix when suitably illuminated.

12. A means of amplifying a target nucleic acid sequence which forms or is capable of forming a hairpin loop structure comprising a single strand of nucleic acid a portion of which can hybridise to another portion of same to produce a single stranded loop structure connected to a partly or wholly double stranded base sequence and detecting the product of said amplification during the reaction, said amplification means comprising;

(i) treating the target strand with at least one first oligonucleotide primer which has at its 3' end a region which is capable of hybridising to the free nucleotides of said loop structure or to the complement of such structure and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents and has at its 5' end a region capable of hybridising to the product of such polymerisation and at least one second oligonucleotide primer which is capable of hybridising to a site on the nucleotide strand upstream of the site of hybridisation of said first primer and of initiating DNA polymerisation from this hybridisation site in the presence of suitable reagents including a reagent that changes its fluorescence characteristics upon interaction with double stranded DNA under conditions such that said hybridisation can occur and such that an extension product will be produced from hybridised primers complementary to their templates and further using an agent for said polymerisation which is capable of displacing downstream hybridised nucleic acid sequences during extension,

(ii) separating the double stranded regions of (i) to render them single stranded,

(iii) repeating the steps of (i) and (ii), and

(iv) monitoring the rate of the reaction by detecting the level of fluorescence emitted from the reaction mix when suitably illuminated.

13. A means as claimed in claim 12 wherein said reagent that changes its fluorescence characteristics does so by intercalation of the reagent into the double stranded DNA.

14. A means as claimed in claim 13 wherein the agent is Ethidium Bromide.

Figure 1

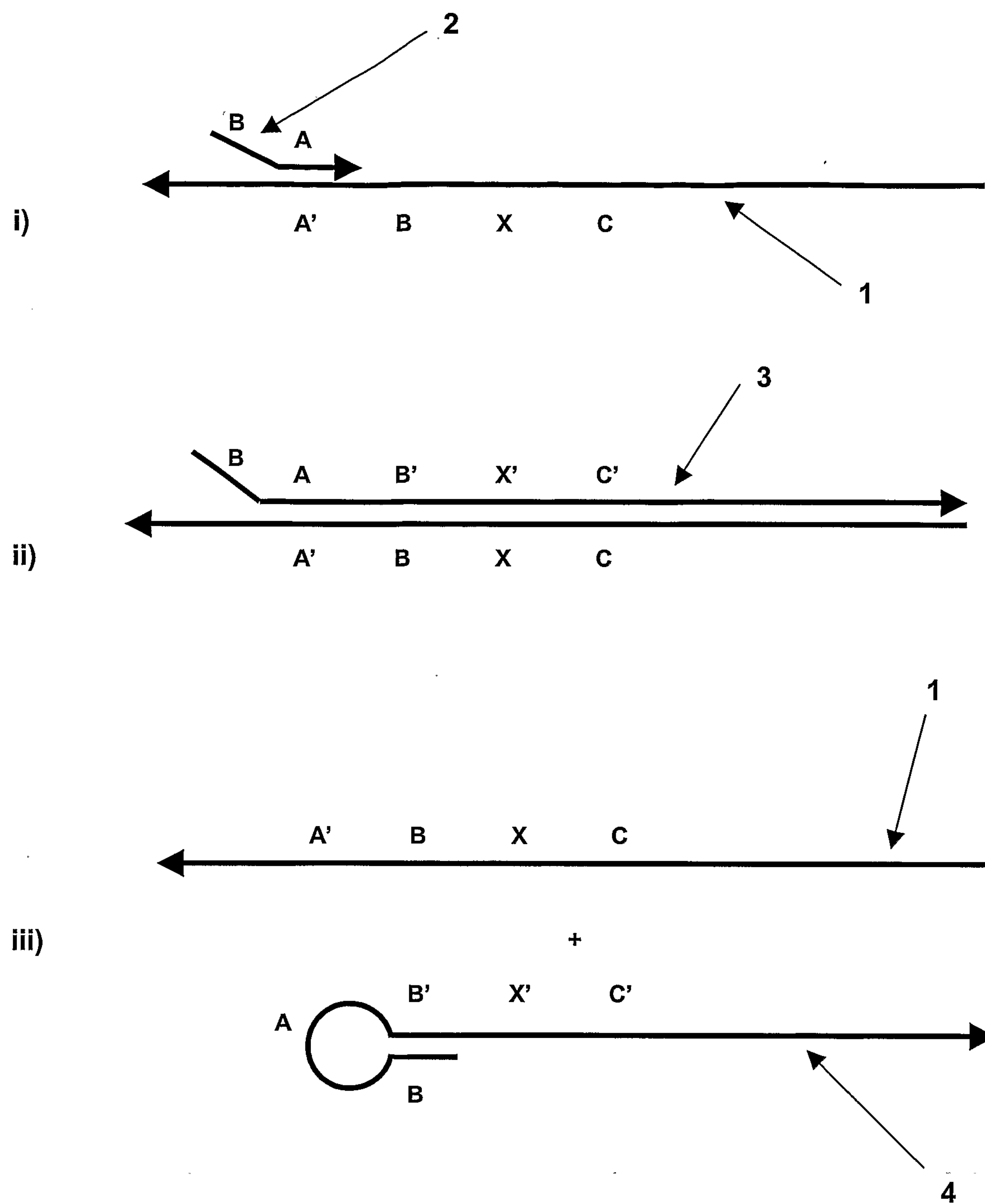


Figure 2

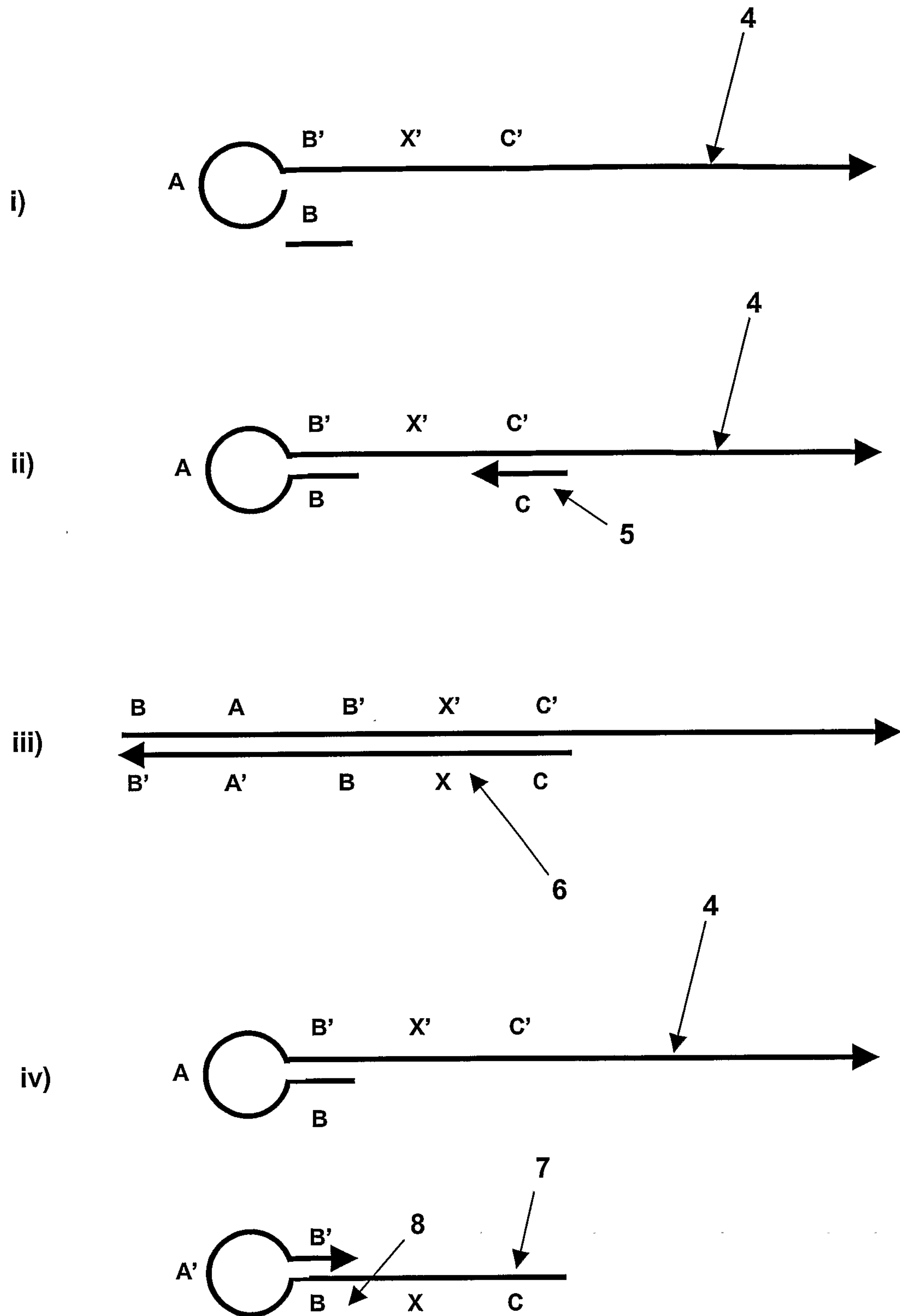
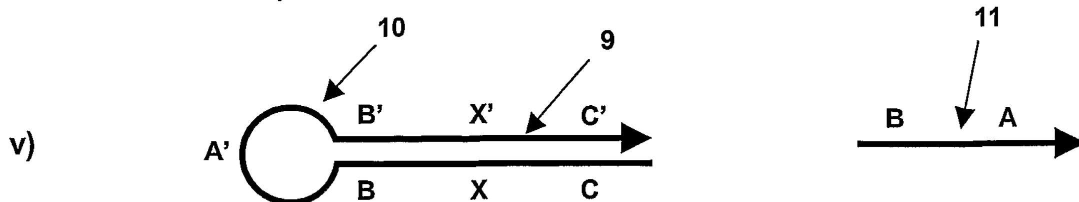
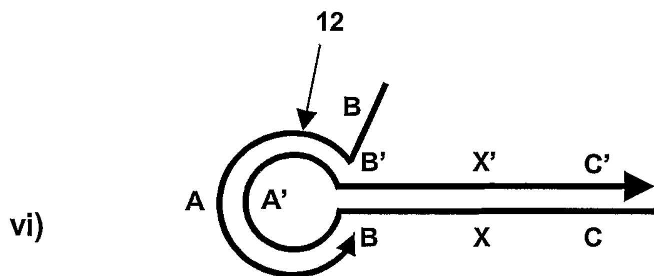


Figure 2 (contd)

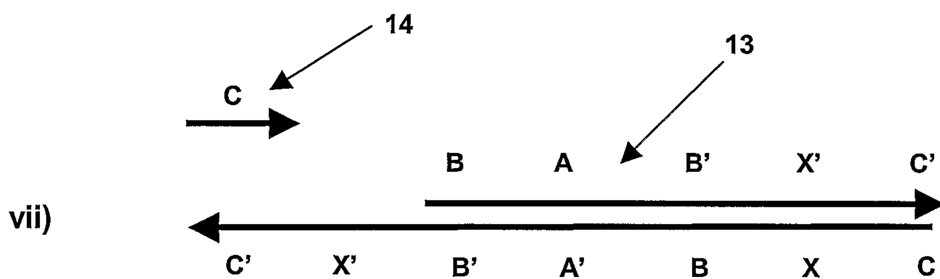
v)



vi)



vii)



viii)

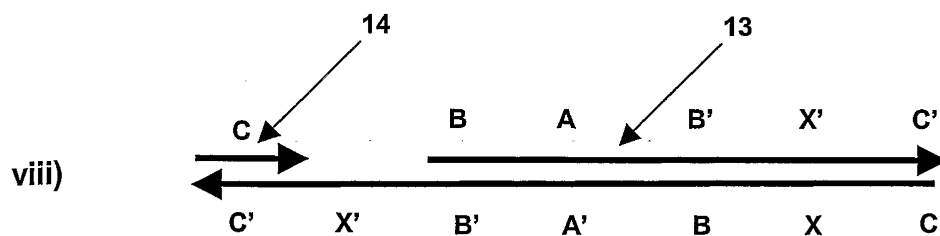
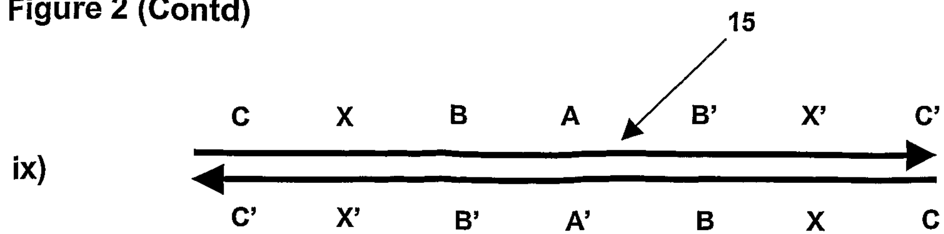
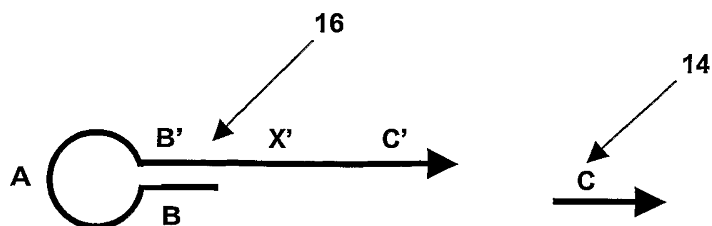


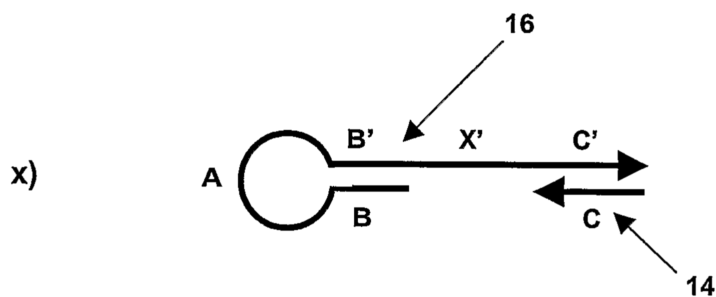
Figure 2 (Contd)

ix)





x)



xi)

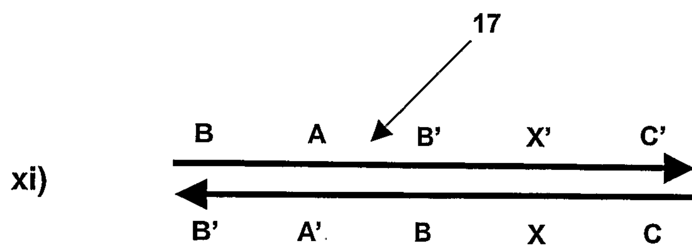


Figure 3

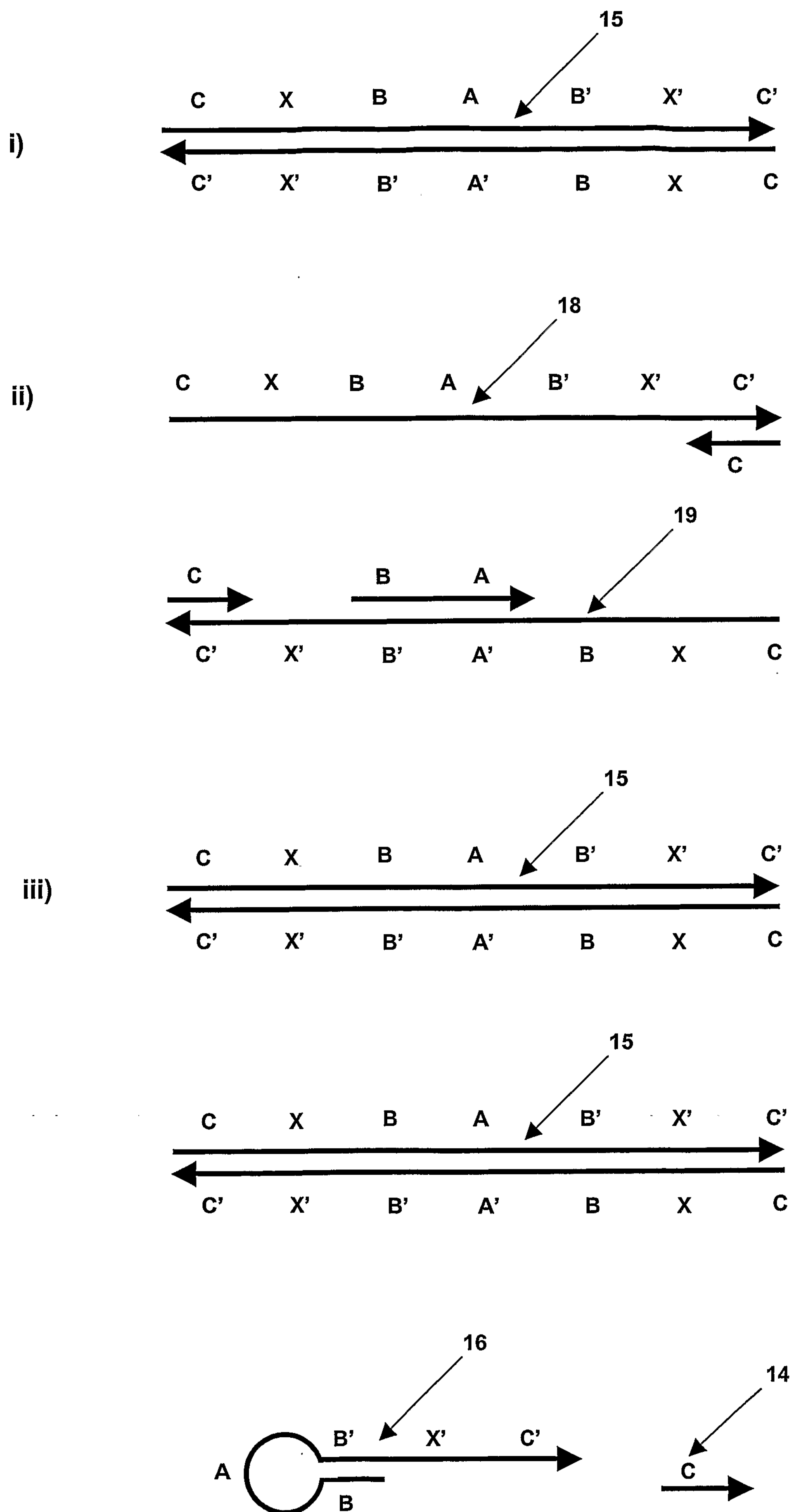
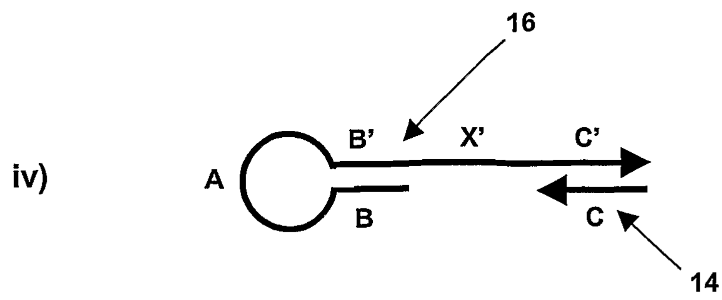


Figure 3 (Contd)

iv)



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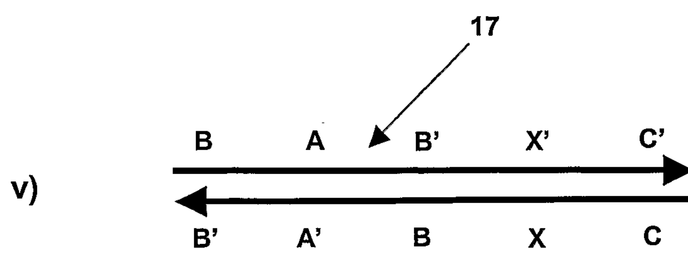
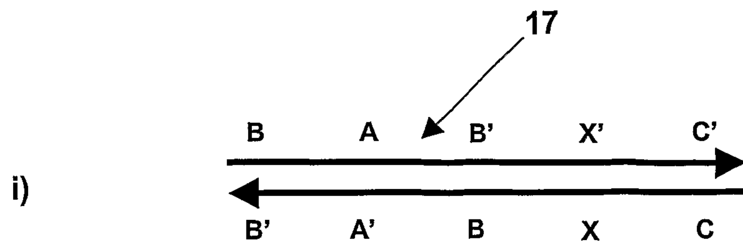
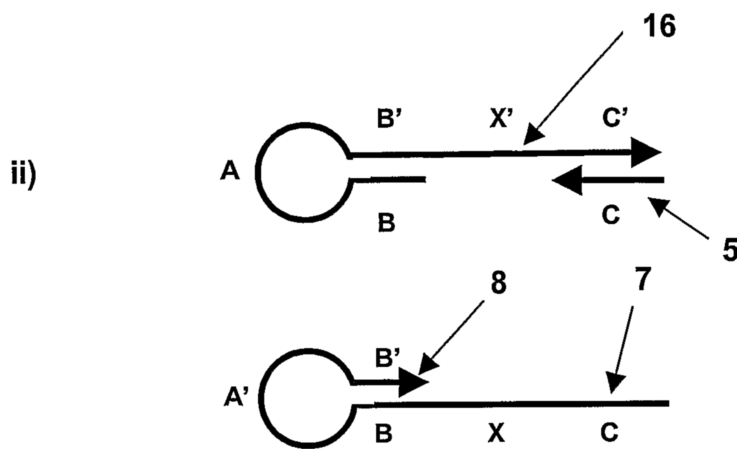
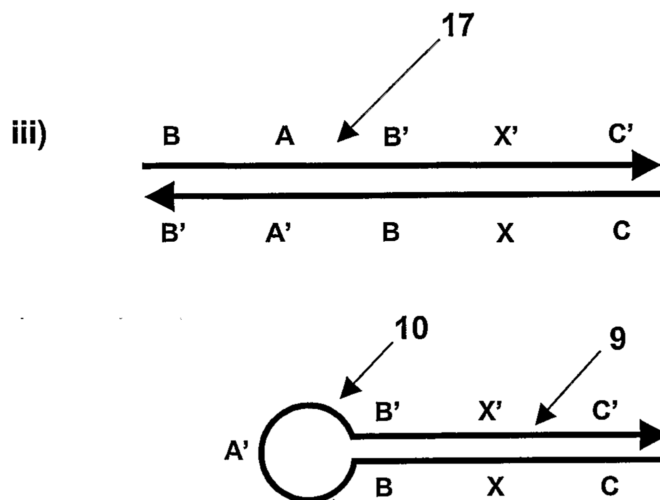


Figure 4



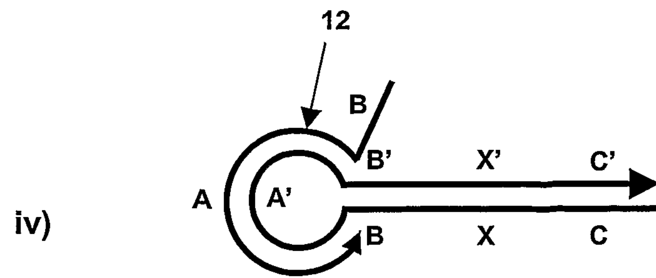




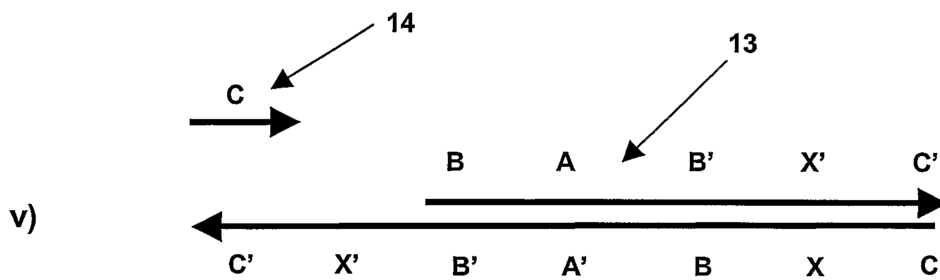
11
B A

Figure 4 (Contd)

iv)



v)



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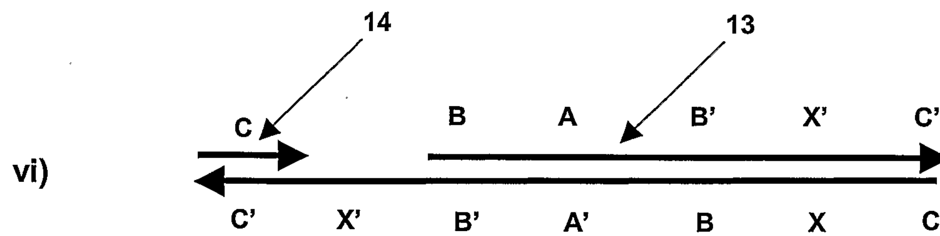

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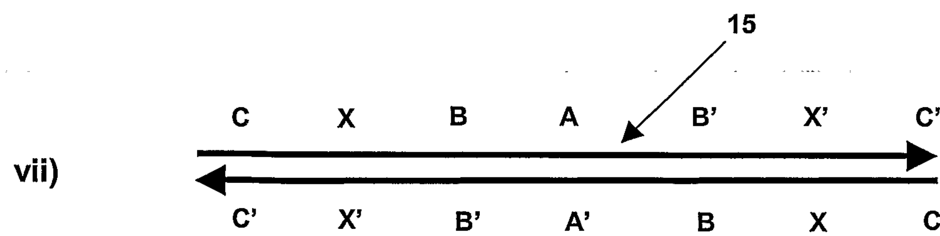

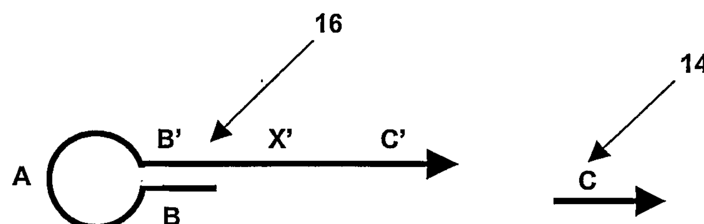

Figure 4 (Contd)

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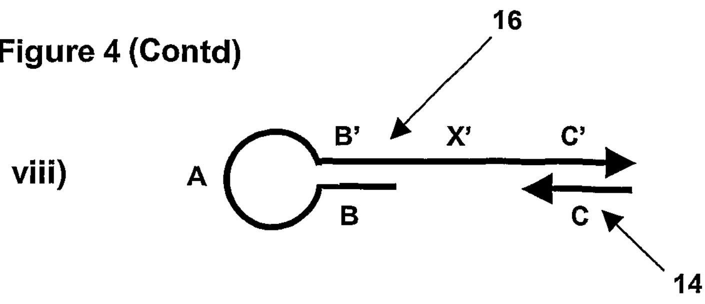

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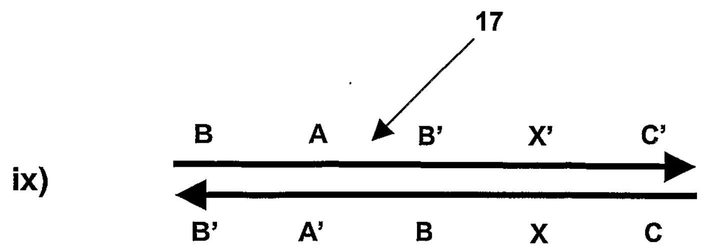


Figure 5

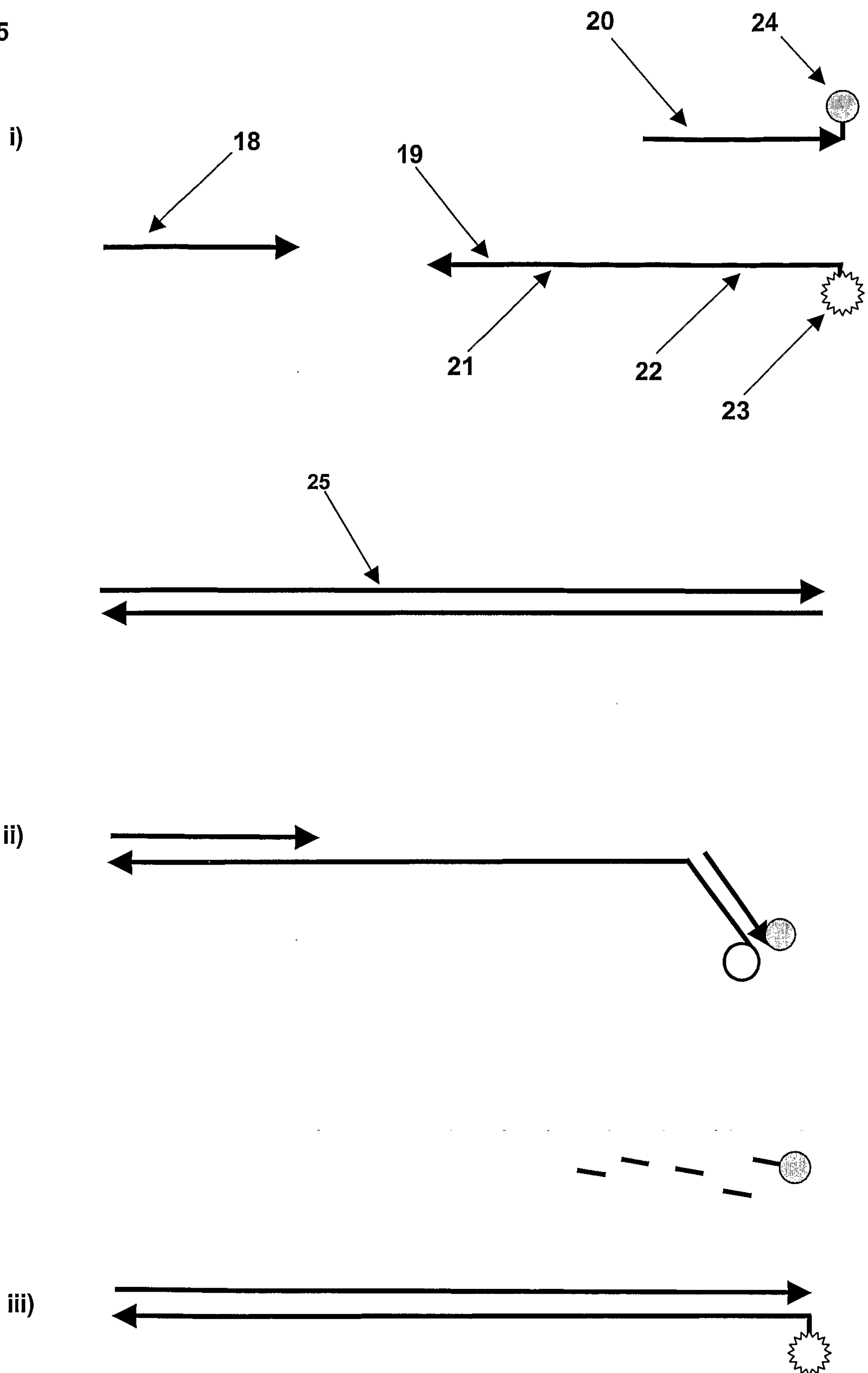
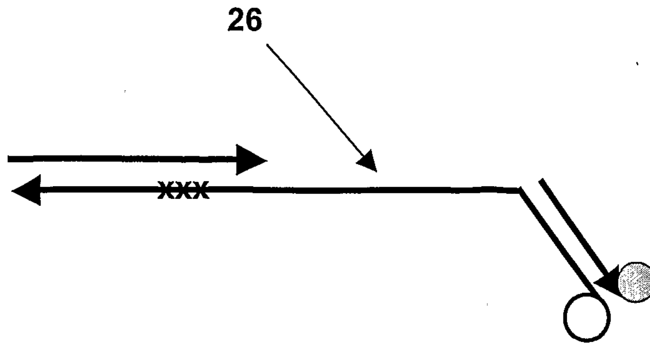


Figure 5 (Contd)

iv



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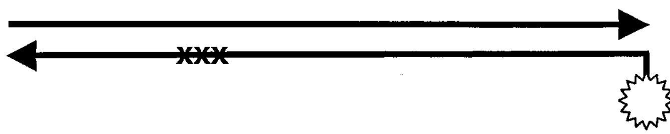


Figure 6a

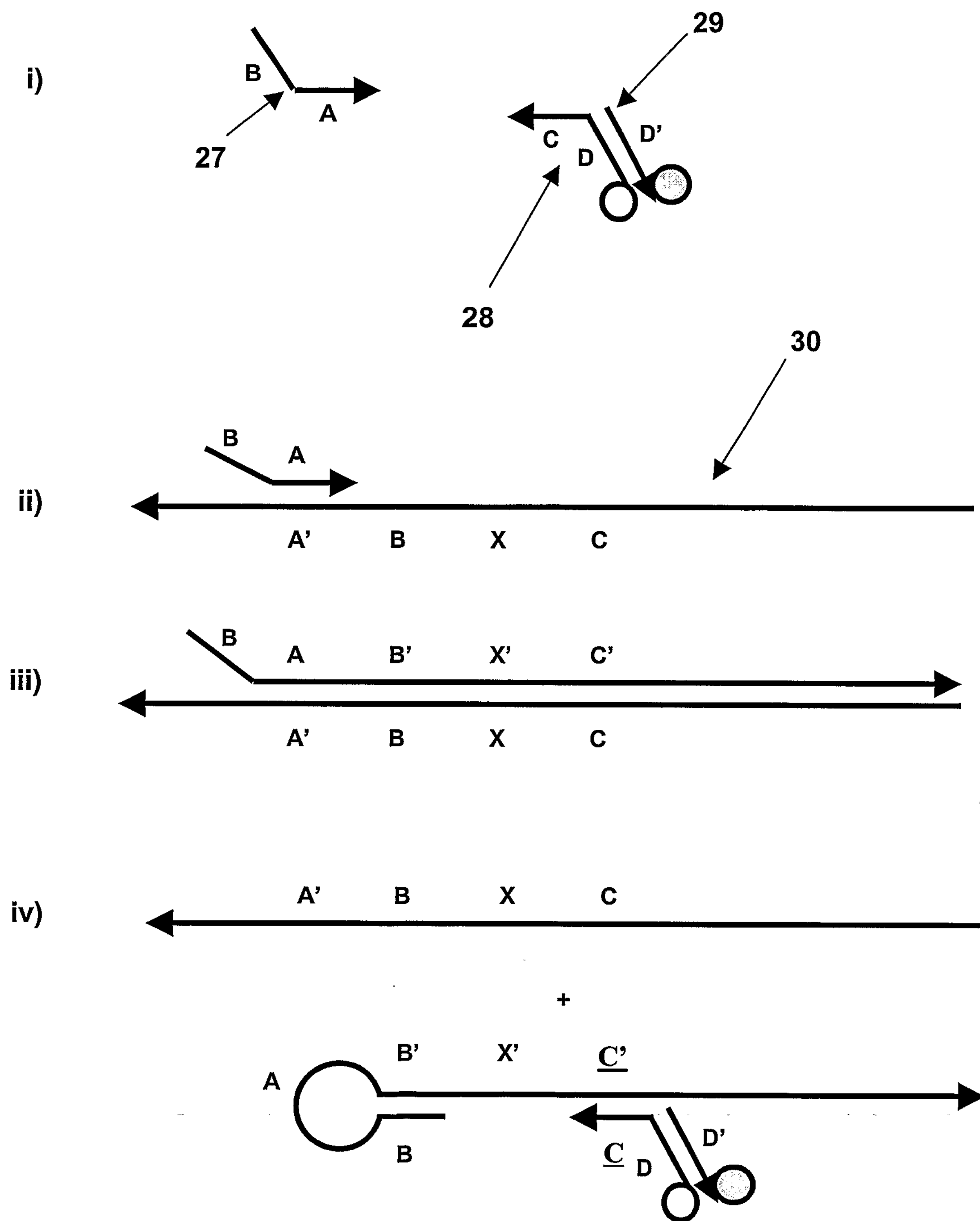
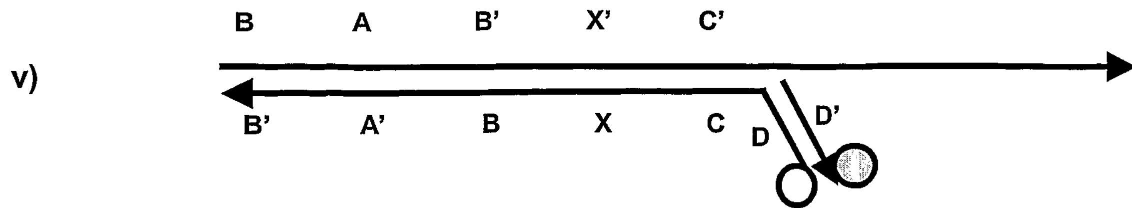


Figure 6a (Contd)

v)



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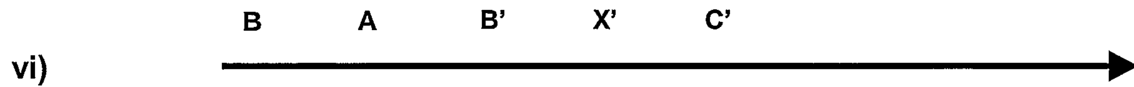

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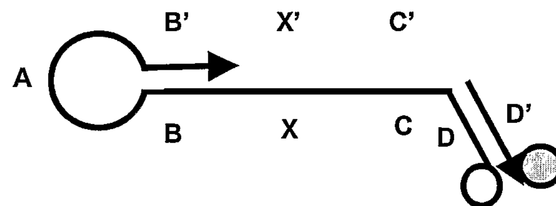

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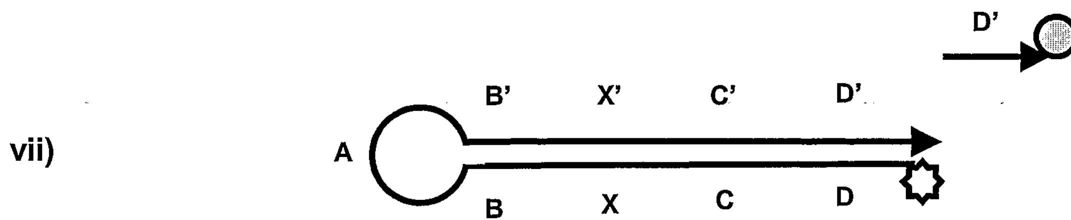


Figure 6b

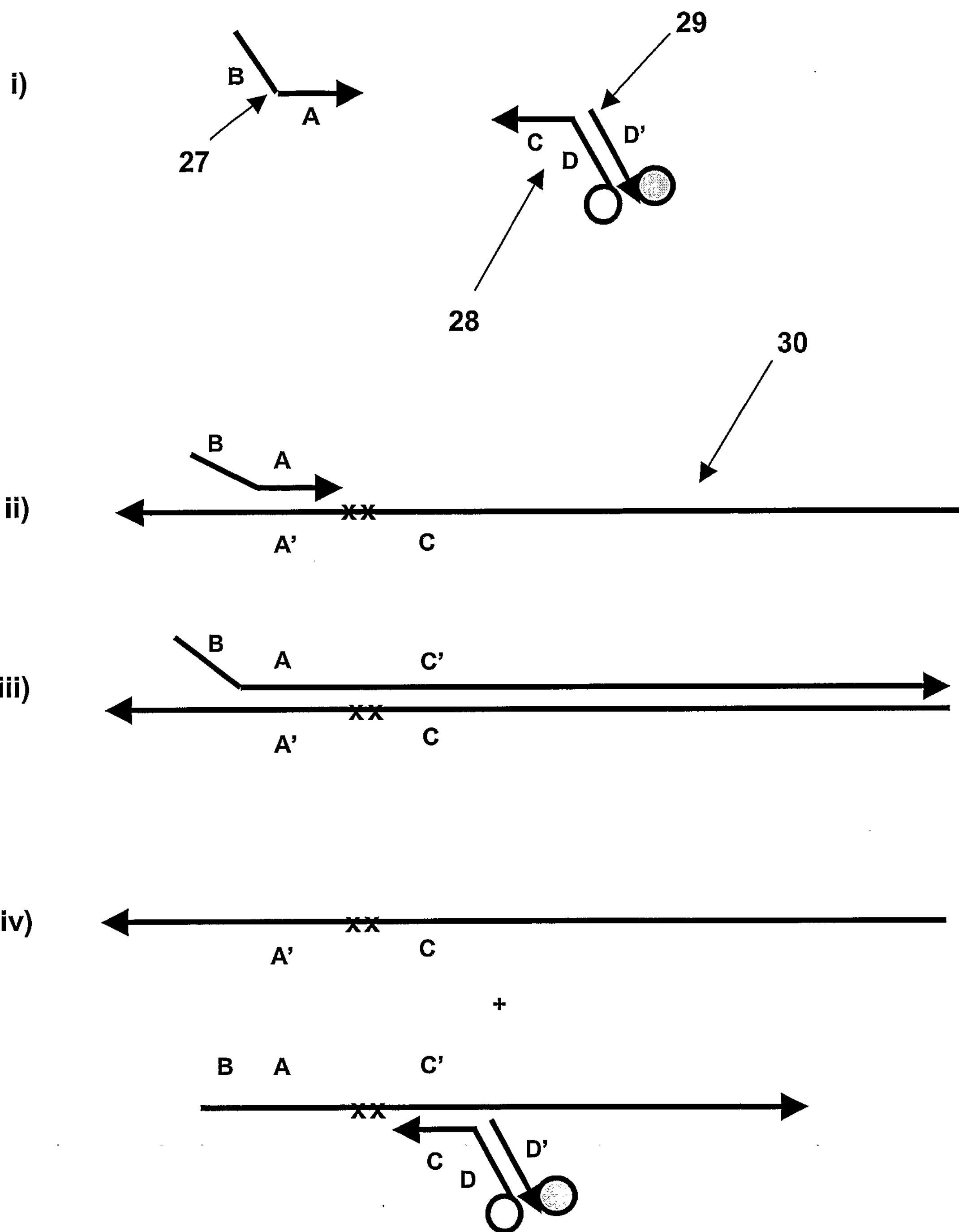


Figure 7

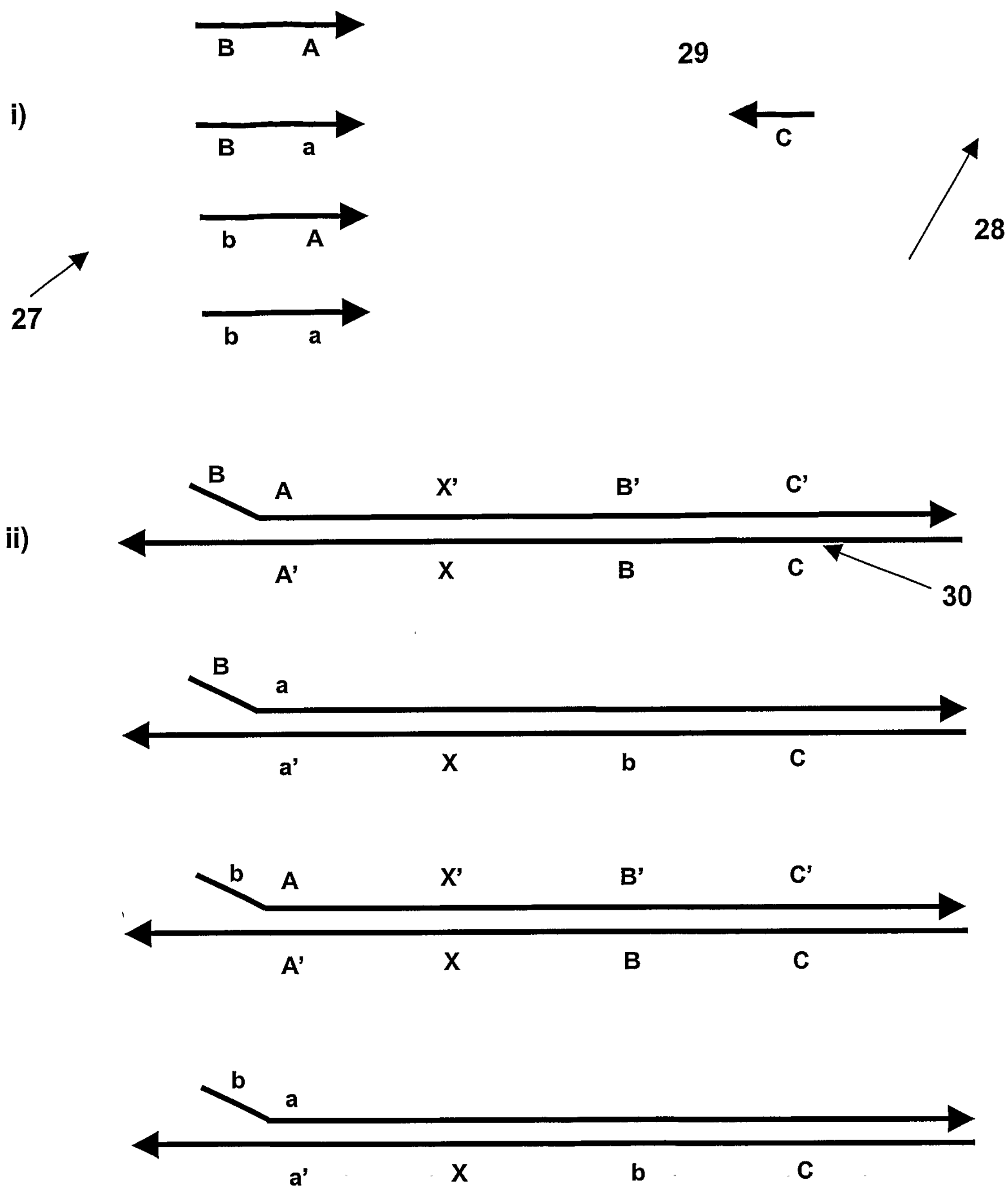


Figure 7 (Contd)

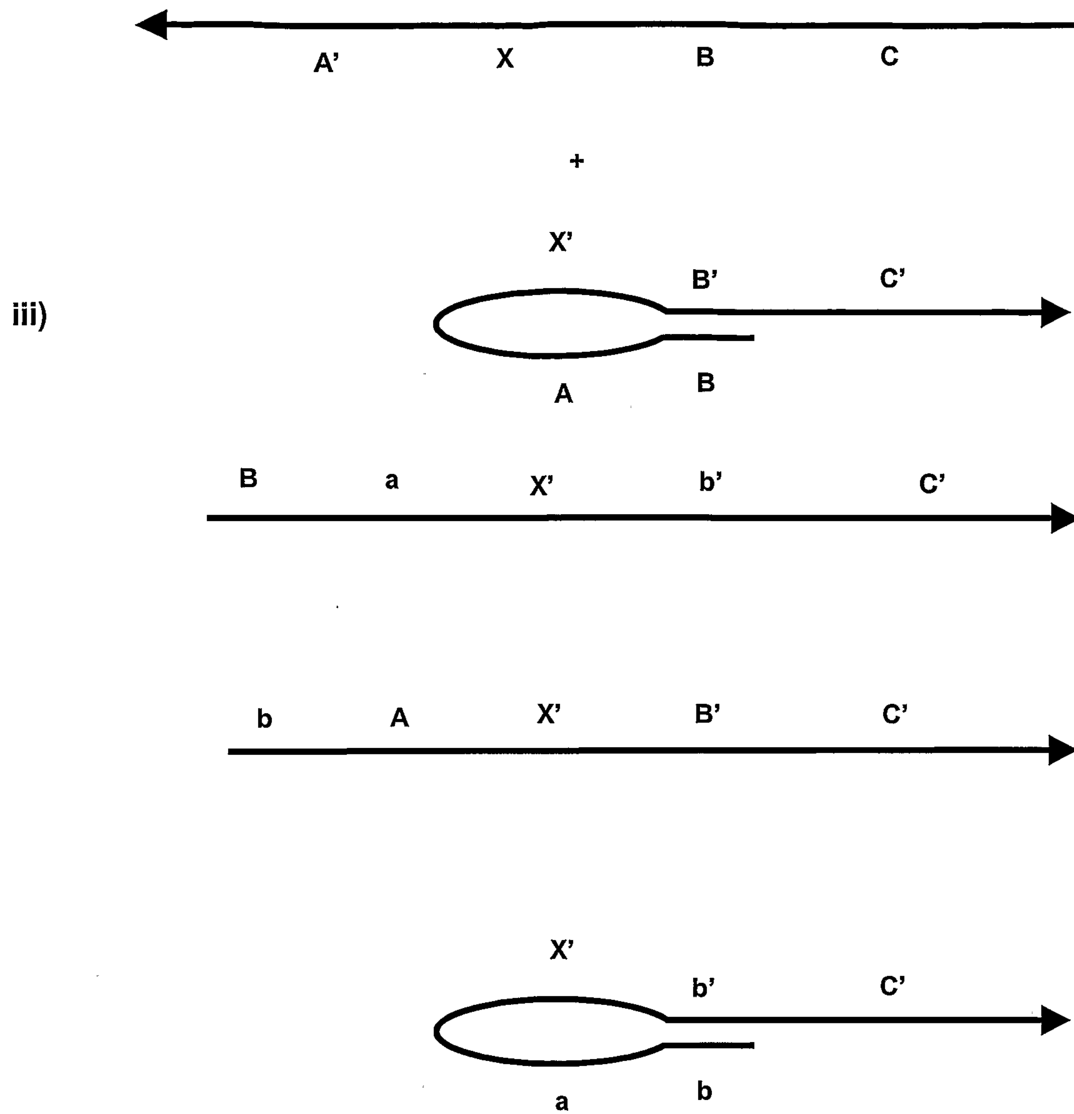


Figure 8

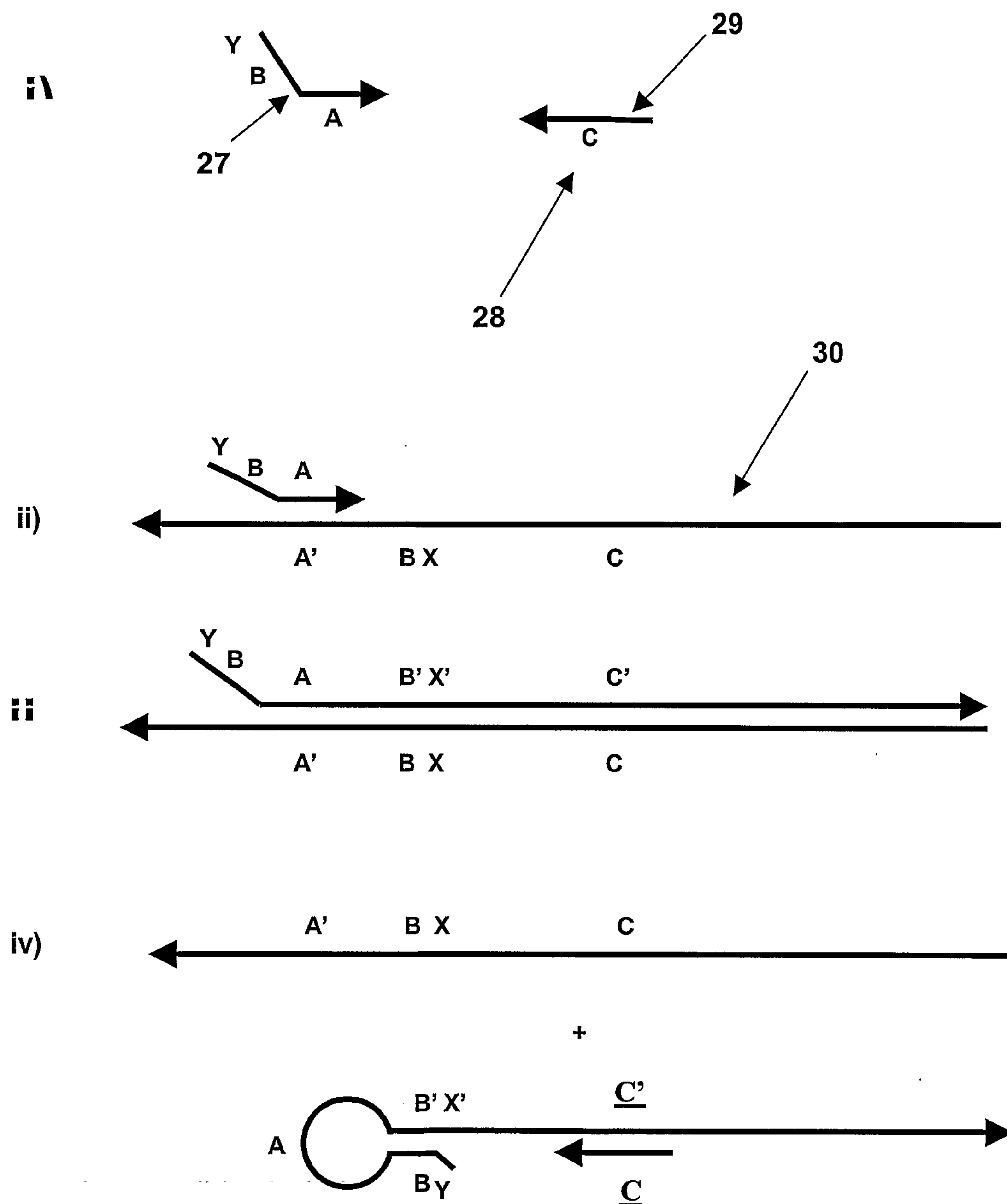
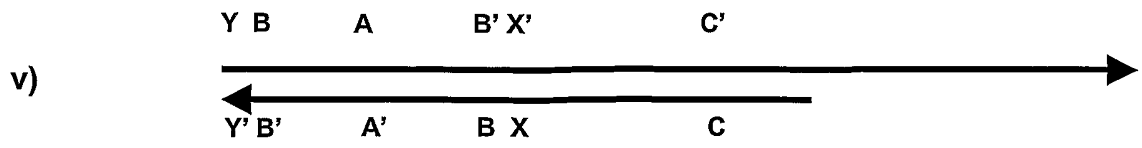


Figure 9

v)



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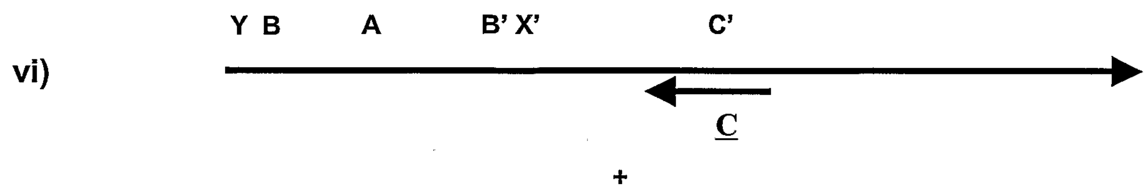

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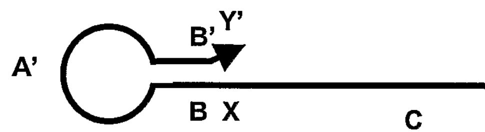

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 03/03422

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

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)
IPC 7 C12Q

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 practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 971 039 A (ENZO DIAGNOSTICS INC) 12 January 2000 (2000-01-12) page 7, line 18 - line 57 page 12, line 55 -page 15, line 20 figures 2,3	1-14
X	US 6 410 278 B1 (HASE TETSU ET AL) 25 June 2002 (2002-06-25) column 12, line 58 -column 13, line 55 column 22, line 6 - line 9 figures 1-6	1-14

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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

"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)

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

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

"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

"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

"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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 November 2003	27/11/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Aguilera, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 34838 A (EIKEN CHEMICAL ;KANDA HIDETOSHI (JP); NAGAMINE KENTARO (JP); NOTOM) 17 May 2001 (2001-05-17) abstract figures 1-4 & EP 1 231 281 A (EIKEN CHEMICAL) 14 August 2002 (2002-08-14) abstract; figures 1-5 page 3, line 50 -page 4, line 51 ---	1-14
X	NOTOMI T ET AL: "LOOP-MEDIATED ISOTHERMAL AMPLIFICATION OF DNA" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 28, no. 12, June 2000 (2000-06), pages I-VII, XP002907443 ISSN: 0305-1048 the whole document ---	1-14
A	US 5 595 891 A (BECKER MARTIN ET AL) 21 January 1997 (1997-01-21) column 4, line 11 -column 6, line 6 column 16, line 1 - line 19 column 16, line 48 -column 18, line 2 column 18, line 61 -column 19, line 25 figure 1; example 1 ---	1-14
A	WO 93 23563 A (CEMU BIOTEKNIK AB ;PETTERSSON BERTIL (SE); UHLEN MATHIAS (SE)) 25 November 1993 (1993-11-25) cited in the application page 2, line 23 -page 4, line 3 figure 1 ---	1-14
A	WILTON ET AL: "SNAPBACK SSCP ANALYSIS: ENGINEERED CONFORMATION CHANGES FOR THE RAPID TYPING OF KNOWN MUTATIONS" HUMAN MUTATION, WILEY-LISS, NEW YORK, NY, US, vol. 11, 1 March 1998 (1998-03-01), pages 252-258, XP002094957 ISSN: 1059-7794 abstract; figure 3 -----	1-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 03/03422

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 1-14 (all partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-14 (all partially)

The large number of independent method claims presently on file, their obscure and repetitive wording, the confusion between the steps of the methods steps and the structural features of the reagents involved, and the definition of said reagents in terms of the effect to be achieved, render it extremely difficult, if not impossible, to determine the matter for which protection is sought. The present application fails to comply with the clarity and conciseness requirements of PCT Article 6 (see also PCT Rule 6.1(a)) to such an extent that a meaningful search on the basis of the claims is impossible.

Consequently, the search has been carried out for those parts of the application which do appear to be clear and concise, namely the method of amplifying nucleic acids as explained in pages 17-25 of the description, specially in regard to Figures 1-4, or, in summary:

Method of amplifying nucleic acids comprising the steps of:
(i) providing a reaction mixture comprising:
  (a) a strand displacing polymerase
  (b) a target nucleic acid strand consisting of sequence segments: 5'-A'-B-X-C-3', wherein X is the sequence to be amplified
  (c) a first primer consisting of sequence segments: 5'-B-A-3', wherein A is complementary to A'
  (d) a second primer consisting of sequence segment C
(ii) effecting repeated temperature dependent cycles of denaturation, hybridization and polymerization.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 03/03422

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