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PATENT REQUEST: STANDARD PATENT

I/We, the Applicant(s)/Nominated Person(s) specified below, request I/We be granted a patent for the invention disclosed in the accompanying standard complete specification.

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[54] Invention Title:
Improved Method for Nucleic Acid Amplification

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Notice Of Entitlement

I, John David O'Connor, of 31 Market Street, Sydney, New South Wales, 2000, Australia, Patent Attorney for the Applicant/Nominated Person in respect of an application entitled:

Improved Method for Nucleic Acid Amplification

state the following:-

The Applicant/Nominated Person has entitlement from the actual inventors as follows:-

The Applicant/Nominated Person is the assignee of the actual inventors.

The Applicant/Nominated Person is the applicant of the basic application listed on the Patent Request.

The basic application listed on the Patent Request is the first application made in a Convention Country in respect of the invention.

Dated 17 August 1993

John David O'Connor

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IMPROVED METHOD FOR NUCLEIC ACID AMPLIFICATION

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(56) Prior Art Documents
AU 41858/89 C07H
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(57) Claim

1. A method for the amplification of a target nucleic acid sequence comprising the steps of:

a) providing a DNA template corresponding to the target sequence including a double stranded promoter recognized by a DNA dependent-RNA polymerase,

b) transcribing multiple copies of RNA from said DNA template using the DNA dependent-RNA polymerase and a mixture of ribonucleotides including nucleotides that weaken normal base pairing,

c) using the generated RNA in which the nucleotides that weaken normal base pairing have been incorporated as a template for the subsequent generation of a new amount of the double stranded template and

repeating steps b and c to amplify the amount of RNA.

IMPROVED METHOD FOR NUCLEIC ACID AMPLIFICATION.

The present invention relates to a method for the amplification of nucleic acid.
The amplification of specific nucleic acid segments is particularly useful for the generation of detectable amounts of certain kinds of nucleic acid. For example when the presence of nucleic acid characteristic for a specific disease state is to be indicated, this nucleic acid is generally present in biological samples in small amounts. To be able to detect these small amounts of nucleic acid either very sensitive detection methods would have to be used or very large amounts of sample material would have to be concentrated. With the present amplification techniques the small amounts of a specific segment of nucleic acid present in a biological sample can be amplified. This amplified nucleic acid can readily be detected by, for example, hybridizing it to a labelled complementary oligonucleotide. Of course the amplification of nucleic acid is also useful for the generation of larger amounts of nucleic acid used in recombinant DNA techniques and for cloning and sequencing purposes.

Known techniques for the amplification of specific nucleic acid segments are, for example, the polymerase chain reaction (PCR), as described in US patent nr. 4, 683,195 and US patent nr. 4,683,202, and Nucleic Acid Sequence Based Amplification (NASBA), as described in European Patent application EP 0,329,822.
With PCR large amounts of deoxyribonucleic acid (DNA) are generated by treating a target DNA sequence with oligonucleotide primers such that a primer extension product is synthesized which is separated from the template using heat denaturation and in turn serves as a template, resulting in the amplification of the target DNA sequence. When RNA is to be amplified with PCR the RNA strand is first transcribed into a DNA strand with the aid of reverse transcriptase. Intermediates in the Polymerase Chain Reaction consist of DNA only.

With the aid of NASBA large amounts of single stranded RNA are generated from either single stranded RNA or DNA or double stranded DNA. When RNA is to be amplified with NASBA the ssRNA serves as a template for the synthesis of a first DNA strand by elongation of a first primer containing s RNA polymerase recognition site. This DNA strand in turn serves as the template for the synthesis of a second, complementary, DNA strand by elongation of a second primer, resulting in a double stranded active RNA-polymerase promoter site, and the second DNA strand serves as a template for the synthesis of large amounts of the first template, the ssRNA, with the aid of a RNA polymerase.

All amplification processes comprise the attachment of primers to templates and subsequent elongation of these primers by certain nucleic acid polymerases that may differ depending on the amplification technique employed.

A problem encountered with amplification of nucleic acid is that nucleic acid is capable of forming various secondary structures. Nucleic acid strands may comprise sequences that may result in the formation of, for example, hairpin loops. These secondary structures might hamper the attachment of a primer to a template and the subsequent elongation of the primer along the template. By interfering with the annealing or extension of the amplification primers these secondary structures lower the efficiency of the amplification.

By the incorporation during amplification of nucleotides that weaken normal base paring the formation of secondary structures, like the formation of internal loops, in the amplificate is prevented. With the incorporation of these structure destabilizing nucleotides secondary structures are destabilized and amplification will become more efficient.

The formation of secondary structures in nucleic acid is also known to be a problem with the sequencing of nucleic acid, because such structures, i.e. compressed regions, may result in anomalous migration patterns during gel electrophoresis. Substitution of inosine for guanosine in the nucleic acid fragments synthesized for the sequencing of RNA has been described by D.R. Mills *et al.*, P.N.A.S., Vol.76, pp-2232-2235. With the introduction of inosine in the nucleic acid fragments secondary structures are prevented and the resolution obtained in gel separations after sequencing of the nucleic acid is thereby improved.

The incorporation of a structure destabilising base analog in DNA amplified with the Polymerase Chain Reaction has been described by Cetus Corporation in PCT application no. WO90/03443. The structure destabilizing nucleotide incorporated during PCR amplification in the method as claimed by Cetus is 7-deaza-2'-deoxyguanosine-5'-triphosphate (c^7dGTP).

The utilization of c^7dGTP in PCR results in the incorporation of 7-deazaguanine into the amplified DNA product. This analog differs from normal guanine in that the N-7 of the guanine ring is replaced with a methine moiety which precludes Hoogsteen bond formation.

For amplification processes with DNA intermediates the incorporation of c^7dGTP increases amplification efficiency.

According to a first embodiment of this invention, there is provided a method for the amplification of a target nucleic acid sequence comprising the steps of:

a) providing a DNA template corresponding to the target sequence including a double stranded promoter recognized by a DNA dependent-RNA polymerase,

b) transcribing multiple copies of RNA from said DNA template using the DNA dependent-RNA polymerase and a mixture of ribonucleotides including nucleotides that weaken normal base pairing,

c) using the generated RNA in which the nucleotides that weaken normal base pairing have been incorporated as a template for the subsequent generation of a new amount of the double stranded template and

repeating steps b and c to amplify the amount of RNA.

According to a second embodiment of this invention, there is provided a kit for the amplification of nucleic acid comprising a mixture of nucleotides, characterised in that the mixture comprises ribonucleotides that weaken normal base pairing.

According to a third embodiment of this invention, there is provided a method for the detection of amplified nucleic acid where the nucleic acid is hybridised to a complementary oligonucleotide, characterised in that the nucleic acid is amplified with a method according to the first embodiment.

The method of the present invention for the amplification of nucleic acid nucleotides is characterized in that ribonucleotides are introduced during amplification that weaken normal base pairing.

Certain RNA sequences are known to form very strong secondary structures that can hardly be disrupted. The ribonucleotides that weaken normal base pairing used with a method according to the present invention are incorporated in ribonucleic acid intermediates during amplification to make the ribonucleic acid less capable of forming secondary structures like hairpin loops that can hinder amplification.

Preferably, as a ribonucleotide that weakens with normal base pairing, inosine-triphosphate (ITP) is used. ITP is an analogue of guanine-triphosphate (GTP). Inosine differs from guanine, when incorporated in nucleic acid, in that it only forms two hydrogen bonds with cytosine whereas guanine forms three hydrogen bonds with cytosine. I-C basepairs therefore are relatively weak compared to normal G-C base pairs. Secondary structures, that are normally held together by the relatively strong G-C basepairs are therefore weakened by the incorporation of ITP or do not form at all. The ITP is incorporated in the ribonucleic acid during primer extension by a suitable nucleic acid polymerase. Since the nucleic acid strands thus obtained are less capable of forming secondary structures, they will serve as more efficient templates in subsequent amplification cycles.

The method according to the invention is especially useful in amplification methods like NASBA that, unlike PCR, generate large amounts of RNA intermediates, starting from DNA as well as RNA.
The incorporation of inosine in DNA intermediates during amplification in PCR results in frequent mismatching of bases during primer extension, as is stated in the above mentioned PCT application of Cetus Corp. (WO 90/03443).
Surprisingly with amplification techniques where RNA intermediates are generated good results are obtained and the error-rate of the amplification is certainly not higher than with conventional amplification, that is without the incorporation of ITP.
Especially when NASBA is used as the amplification technique, the incorporation of ITP during amplification might even result in a 10-100 times higher amplification factor. This means that, with the incorporation of ITP, 10-100 times more RNA will be produced from the originally amount of nucleic acid present in a biological sample than without the incorporation of ITP.

With the method according to the invention ITP can be added to the amplification mixture containing normal ribonucleotides like GTP, UTP, CTP and ATP.

Preferably the ITP added partly substitutes the GTP normally present. Good results are obtained when no more than 50% of the GTP present in a amplification reaction mixture is substituted with ITP. When the ratio of ITP:GTP is to high the amplification is hampered. ITP has proven to be not as good as a substrate for the enzymes used with amplification, nucleic acid polymerases like the T7polymerase used with NASBA, as normal GTP. The optimal ratio of ITP:GTP has been found to be approximately 1:3. In this way enough ITP will be incorporated in the amplified nucleic acid to interfere with the formation of rigid secondary structures while amplification is not yet hindered by the amount of ITP present in the amplification reaction mixture.

A kit for the amplification of nucleic acid, comprising a mixture of nucleotides characterized in that the mixture comprises ribonucleotides that weaken normal basepairing, is also part of the present invention. Such an amplification kit may further comprise suitable amplification primers for the specific nucleic acid to be amplified and other amplification reagents like the necessary enzymes.

The present invention also relates to a method for the detection of amplified nucleic acid where the nucleic acid is hybridized to a complementary detection probe and the nucleic acid to be detected was amplified with the method for the amplification of nucleic acid according to the invention. The incorporation of nucleotides that weaken normal basepairing during amplification results in both a more efficient amplification and a more sensitive detection. The hybridization of a complementary detection probe to the nucleic acid that is to be detected can also be affected by the presence of secondary structures in the amplified nucleic acid. The secondary structures can hinder the oligonucleotide in its binding to the amplified nucleic acid. The interference of secondary structures with the binding of a labelled oligonucleotide to the nucleic acid may reduce the sensitivity of the detection method. With the method for the detection of amplified nucleic acid according to the invention sensitivity is increased because the formation of secondary structures that interfere with the annealing of amplified nucleic acid and a complementary oligonucleotide is prevented. The advantage of the incorporation of the interfering nulceotides in the amplified nucleic acid therefore is twofold: not only the efficiency of the amplification is increased but also the sensitivity of detection methods, where nucleic acid is detected by hybridizing it to a detection probe, is markedly improved.

One common way in which the detection of amplified nucleic acid is often carried out is by subjecting the sample with amplified nucleic acid to gel electrophoresis, blotting the gel onto a filter and hybridizing the nucleic acid with a detection probe, where the detection probe is a labelled complementary oligonucleotide.
Nucleic acid to be detected that is amplified with the method according to the invention will be bound to the filter in a rather unwinded form because the formation of secondary structures is prevented by the incorporation of nucleotides that weaken normal basepairing. Because of the absence of secondary structures hybridization of a complementary sequence to the amplified nucleic acid present on a filter, or on any other solid phase, is improved.
Of course the detection method according to the invention is not limited to the above described embodiment. Any detection method involving hybridization of the amplified nucleic acid to a complementary sequence will benefit from the effects of the incorporation of nucleotides that weaken normal base pairing during amplification whereby the formation of secondary structures is prevented. The method according to the invention can equally well be applied to any other detection method where the amplified nucleic acid is hybridized to a complementary sequence. For example, the detection probe can also be a complementary oligonucleotide immobilized on a solid phase, like in a sandwich hybridization assay. In this

case the amplified nucleic acid is bound to the solid phase by hybridizing to the complementary oligonucleotide immobilized on the solid phase and can be detected by hybridizing the immobilized amplified nucleic acid with a second, labelled, complementary oligonucleotide.

Furthermore the present invention relates to a testkit for the detection of amplified nucleic acid. Such a testkit may comprise suitable reagents for the amplification of the nucleic acid including a mixture of nucleotides comprising a certain amount of nucleotides that weaken normal basepairing, suitable amplification primers and enzymes (nucleic acid polymerases) and detection means like, for example, a solid phase with complementary oligonucleotides immobilized thereon to which the amplified nucleic acid can be bound and a second complementary labelled oligonucleotide.
Or, when the amplified nucleic acid is subjected to electrophoresis, suitable reagents, for detection of the nucleic acid after electrophoresis is completed, can be included in the testkit. Reagents for detection of the amplified nucleic acid after electrophoresis may comprise a labelled complementary oligonucleotide which may be hybridized to the amplified nucleic acid before or after electrophoresis. When the oligonucleotide is enzyme labelled the testkit may also comprise a suitable substrate for the enzyme label.

A possible embodiment of the present invention is given in Example 1. From this example, and figure 1 in which the results are depicted, it can be seen that incorporating ITP in the amplification procedure markedly improves the efficiency of the amplification procedure and results in a higher sensitivity during detection. The intensity of the signal obtained during detection is increased because of the improved binding of the labelled oligonucleotide to amplificate.

EXAMPLES:

Example 1: Amplification of part of the HCV genome with NASBA and subsequent detection of amplified nucleic acid.

Vector pGem7z f(+) (Promega) contains an insertion in the sma I site (multiple cloning site), of 277 nt (UTR-region) of the HCV genome. The vector was called #14.
Transcription with T7-polymerase was performed according to the Promega protocol, thereby generating the (+) RNA strand.
In vitro generated (+)RNA was amplified according to the (standard) NASBA-sop.
ITP was added to the 2.5 NM NASBA buffer, the ITP:GTP ratio being 1:3.
The amplified nucleic acid was subjected to electrophoresis on a native gel system (3% nusive/ 1%agarose) and blotted onto zetaprobe membrane and hybridized to ^{32}P labelled oligonucleotides complementary to an internal fragment of the amplificate.
Samples with different starting amounts of nucleic acid molecules (inputs varying from 10^2 to 10^8 molecules) were subjected to amplification.
The results obtained were compared to the results obtained when the same nucleic acid segment was amplified without the addition of ITP.
The results are given in figure 1. From this figure it can be seen that 10^2 molecules input of nucleic acid amplified in the presence of ITP resulted already in a detectable band in the gel, whereas nucleic acid amplified in the absence of ITP only gave a detectable signal when amplification was started with 10^4 molecules input.

The claims defining the invention are as follows:

1. A method for the amplification of a target nucleic acid sequence comprising the steps of:

   a) providing a DNA template corresponding to the target sequence including a double stranded promoter recognized by a DNA dependent-RNA polymerase,

   b) transcribing multiple copies of RNA from said DNA template using the DNA dependent-RNA polymerase and a mixture of ribonucleotides including nucleotides that weaken normal base pairing,

   c) using the generated RNA in which the nucleotides that weaken normal base pairing have been incorporated as a template for the subsequent generation of a new amount of the double stranded template and

   repeating steps b and c to amplify the amount of RNA.

2. Method according to claim 1, characterised in that the ribonucleotides are inosine-triphosphate nucleotides.

3. Method according to claim 2, characterised in that the inosine-trisphosphate nucleotides partly substitute guanine-triphosphate nucleotides.

4. Method according to claim 3, characterised in that inosine-triphosphate nucleotides are substituted for no more than 50% of the guanine-triphosphate nucleotides.

5. Kit for the amplification of nucleic acid comprising a mixture of nucleotides, characterised in that the mixture comprises ribonucleotides that weaken normal base pairing.

6. Method for the detection of amplified nucleic acid where the nucleic acid is hybridised to a complementary oligonucleotide, characterised in that the nucleic acid is amplified with a method according to any one of claims 1-4.

7. Testkit for the detection of nucleic acid, characterised in that the nucleic acid is detected according to the method of claim 6.

8. Method for the amplification of a target nucleic acid sequence, which method is substantially as hereinbefore described with reference to Example 1.

9. Method for the detection of amplified nucleic acid where the nucleic acid is hybridised to a complementary oligonucleotide, which method is substantially as hereinbefore described with reference to Example 1.

Dated 3 June, 1996
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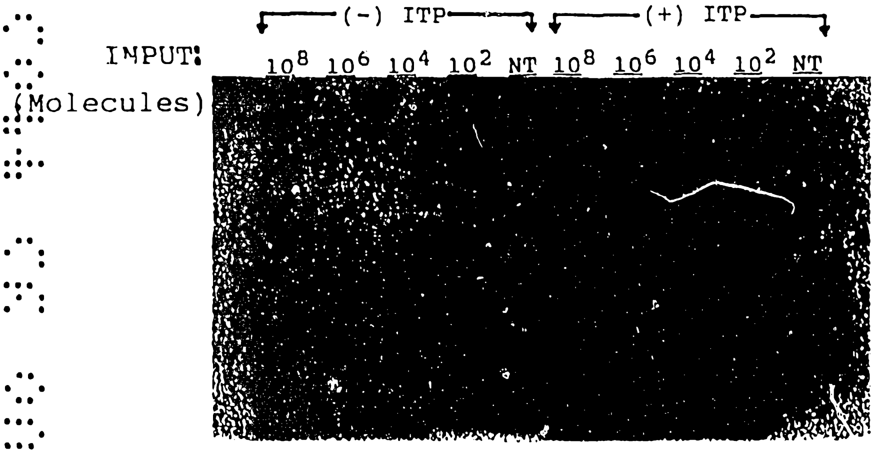
IMPROVED METHOD FOR NUCLEIC ACID AMPLIFICATIION

ABSTRACT

The present invention relates to an improved method for the amplification of nucleic acid. The invention is characterized in that ribonucleotides are introduced during amplification that weaken normal basepairing. Preferably the ribonucleotides are inosine-triphosphate nucleotides which partly substitute guanine-triphosphate nucleotides normally present in the amplification reaction mixture. The incorporation of nucleotides, during amplification, that weaken normal base pairing prevents the formation of secondary structures in the amplificate. The efficiency of the amplification is thereby increased. The introduction, during amplification, of nucleotides that weaken normal base pairing also results in an improved sensitivity during detection of the amplified nucleic acid, when the detection method comprises the hybridization of the amplified nucleic acid to a complementary sequence.

FIGURE 1:



NT = no template