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(54) Title: ENZYMATIC INCORPORATION OF LNA NUCLEOTIDES

(57) Abstract: The present invention relates to oligonucleotides comprising LNA units and enzymatic methods of synthesizing such oligonucleotides. Oligonucleotide comprising LNA units have various characteristics, e.g. improved biostability and increased affinity for complementary oligonucleotides. The methods may be part of detection reactions, polymerase chain reaction, RNA transcription or in vitro evolution processes such as SELEX.

ENZYMATIC INCORPORATION OF LNA NUCLEOTIDES

Field of the invention

The present invention relates to nucleic acids and oligonucleotides comprising
5 non-natural units, in particular LNA units (locked nucleic acids).

Background of the invention

Oligonucleotides are used in many aspects of science and technology. They may
e.g. be used as probes for detection of other nucleic acids. Two representative
10 examples are probes on a microarray and probes used for northern/southern
blotting.

Oligonucleotides are also used as reagents, i.e. as primers in PCR, where they
facilitate not only amplification of nucleic acids, but also manipulations such as
15 introduction of restriction sites, point mutations etc. in the PCR products.

There is also great interest in developing oligonucleotides as new therapeutics.
Examples are gapmers that mediate RNase H inactivation of target mRNAs,
siRNAs that guide RISC mediated degradation of target mRNAs and antimirs (also
20 termed antagomirs) that inactivate microRNAs by an antisense mechanism. The
aforementioned all interact with their target via base pairing.

Another class of oligonucleotides that are of interest as therapeutics is so-called
aptamers. Aptamers are typically identified using an in vitro evolution process
25 termed SELEX (systematic enrichment of ligands by exponential enrichment) and
bind to their target by way of their three-dimensional structure.

For all the above classes of oligonucleotides, an important parameter is the base
pairing characteristics (intra- or intermolecularly). Thus, it is very often desirable
30 that base pairing is strong and specific. To this end, various modifications and

non-natural nucleotides have been developed. A very successful non-natural nucleotide is the one called LNA (locked nucleic acid). LNA nucleotide monomers are nucleic acid analogues with a fixed C3'-endo/N-type furanose conformation that mimics the ribose conformation of A-type helical RNA. Incorporation of one or
5 more LNA units into a double stranded nucleic acid dramatically increases the melting temperature of the complex. Hence, LNA allows the use of e.g. shorter probes in detection assays and also shorter therapeutic oligonucleotides.

Another very important feature of LNA is that it improves the biostability of
10 oligonucleotides, i.e. oligonucleotides comprising LNA are less prone to degradation by nucleases. This is not surprising, since LNA is an artificial oligonucleotide building block and the nucleases have not evolved to recognize non-natural building blocks.

15 For the same reason, LNA comprising oligonucleotides have hitherto been synthesized using chemical syntheses. I.e. natural polymerases would not be expected to be capable of incorporating non-natural LNA units and/or extending incorporated LNA units.

20 LNA is described in US 6,794,499. Enzymatic incorporation of LNA into DNA was attempted using Klenow fragment DNA polymerase I. It was found that addition of a first LNA unit was quite efficient, but that addition of a second consecutive LNA unit was very inefficient. Hence, the results provide no motivation for attempting syntheses of further LNA units and there still remains a need for enzymatic
25 synthesis of oligonucleotides comprising LNA.

Latorra et al, 2003 studied the effects of LNA substitution in PCR primers and found advantages for the best LNA primer designs.

30 Levin et al, 2006 also studied the effects of LNA substitution in PCR primers as well as sequencing primers. They found favorable effects of LNA substitution near the 5'end of the primers.

Schmidt et al, 2004 studied post-SELEX introduction of LNA monomers into aptamers to improve in vivo stability of aptamers without compromising binding affinity.

- 5 Veedu et al., 2007 described first strand synthesis with incorporation of LNA units, but not second strand synthesis or PCR. The authors are also inventors of the present invention.

Summary of the invention

- 10 In a first aspect, the present invention relates to a method of enzymatic synthesis of an oligonucleotide comprising an LNA unit. A second aspect of the invention is a kit comprising a polymerase, a nucleotide mix and a LNA nucleotide. Other aspects are use of a LNA nucleotide for PCR, use of a LNA nucleotide for transcription and use of a polymerase for the preparation of an oligonucleotide
15 comprising a LNA unit.

Detailed description of the invention

Brief description of the figures.

- 20 Figure 1.
pfu DNA polymerase incorporation of LNA-T.

Figure 2.
Phusion™ High Fidelity DNA polymerase incorporation of LNA-T.

- 25 Figure 3.
Phusion™ High Fidelity DNA polymerase incorporation of LNA-A.

- Figure 4.
30 Phusion™ High Fidelity DNA polymerase multiple incorporation of LNA.

Figure 5.

Phusion™ High Fidelity DNA polymerase successive incorporations – template design.

Figure 6.

5 Phusion™ High Fidelity DNA polymerase successive incorporations – results.

Figure 7.

Checking the fidelity using LNA-TTP.

10 Figure 8.

Checking the fidelity using LNA-ATP.

Figure 9.

Phusion™ High Fidelity DNA polymerase incorporation of Amino-LNA-T.

15

Figure 10.

Phusion™ High Fidelity DNA polymerase multiple incorporation of LNA on LNA modified template.

20 Figure 11

Incorporation of LNA-A by Terminator™ Polymerase.

Figure 12

Incorporation of LNA-T, a comparison of two polymerases.

25

Figure 13

Incorporation of LNA-A by 9° North DNA Polymerase.

Figure 14

30 Incorporation of both LNA-T & A by 9° North DNA Polymerase.

Figure 15

PCR using Phusion polymerase – experimental design.

35 Figure 16

PCR using Phusion polymerase – experimental design.

Figure 17

PCR using Phusion polymerase – experimental design.

5

Figure 18

PCR using Phusion polymerase – experimental design.

Figure 19

10 PCR using Phusion polymerase – results.

Figure 20

PCR using Phusion polymerase – experimental design.

15 Figure 21.

PCR using Phusion polymerase – results.

Figure 22.

Comparitive study of phusion and 9 ° North – experimental design.

20

Figure 23.

Comparitive study of phusion and 9° North – results.

Figure 24.

25 PCR using LNA-ATP using 9° North DNA polymerase.

Figure 25.

PCR results - Using LNA-T and 9N Polymerases.

Figure 26.

30 PCR amplification using LNA-A and LNA-T

Figure 27.

PCR experiment without annealing step using LNA-ATP and 9 °N polymerase

35 Figure 28

Incorporation of N-methyl amino LNA-TTP with Phusion polymerase

Figure 29.

Incorporation of N-methyl amino LNA-TTP with 9 ° North polymerase

5

Figure 30.

Phusion polymerase is inefficient in incorporating LNA-T.

Figure 31.

10 Phusion polymerase is enables efficient incorporation of N-acetyl amino LNA-T.

Figure 32.

9 ° North polymerase enables efficient incorporation of N-acetyl amino LNA-T

15 Figure 33.

Phusion polymerase enables efficient incorporation of amino LNA-T.

Figure 34.

9 ° North polymerase enables incorporation of amino LNA-T.

20

Figure 35.

Phusion polymerase enables efficient incorporation of amino LNA-T.

Figure 36.

25 9 ° North polymerase enables incorporation of amino LNA-T.

Figure 36.

9 ° North polymerase enables incorporation of amino LNA-T.

30 Figure 37.

The efficiency of LNA-T incorporation by Phusion polymerase improves by increasing the reaction time.

Figure 38.

The efficiency of LNA-T incorporation by 9 ° North polymerase is not significantly affected by the reaction time.

Figure 39.

- 5 Phusion polymerase is inefficient with regards to incorporation of N-acetyl amino LNA-T.

Figure 40.

- 9 ° North polymerase is inefficient regards to incorporation of N-acetyl amino
10 LNA-T.

Figure 41.

Checking the fidelity using amino LNA-T.

- 15 Figure 42.

Phusion polymerase enables incorporation of LNA-T on LNA modified template.

Figure 43.

Phusion polymerase enables incorporation of LNA-T and LNA-A.

- 20

Figure 44.

Incorporation of LNA-A into a RNA transcript using T7 RNA polymerase.

Lane 1: using LNA-ATP instead of rATP, lane 2: using all natural rNTPs, lane 3:
27n control RNA, lane 4: primer, 19n.

- 25

Figure 45.

KOD DNA polymerase enables incorporation of LNA-T, LNA-A and LNA-5-methyl-C
together with DNA-G nucleotides using a DNA template.

- 30 Figure 46.

KOD DNA polymerase catalyzes successive incorporation of LNA-5-methyl-C
nucleotides using a DNA template.

Figure 47.

KOD DNA polymerase catalyzes incorporations of LNA-T, LNA-A and LNA-5-methyl-C nucleotides from a LNA-only mix using a DNA template.

Figure 48

5 PCR using KOD DNA polymerase – experimental design.

Figure 49

PCR using KOD DNA polymerase – results.

10 Figure 50

MALDI-TOF MS analysis of the PCR products.

Figure 51.

Incorporation of LNA-U into a RNA transcript using T7 RNA polymerase.

15

Disclosure of the invention

The present invention provides methods for enzymatic synthesis of oligonucleotides comprising an LNA unit. As described in the background section, such methods have not previously been available. Not all polymerases and
20 conditions allow enzymatic incorporation of LNA nucleotides, but the examples section identifies several polymerases and conditions that does. And the skilled man will be motivated and capable of designing further experiments in line with those shown in the examples to identify other polymerases and conditions that enable incorporation of LNA nucleotides. Such work will not require inventive skill
25 or undue burden of the skilled man.

The methods of the present invention have several advantages. First, the method may be used to improve the yield when synthesizing a particular oligonucleotide. Second, the product of the enzymatic synthesis will have an improved stability
30 (i.e. reduced degradation) which is of interest e.g. when performing PCR on a sample that contains nucleases that degrades the template and/or the PCR product. Third, oligonucleotides comprising LNA have a variety of uses and enzymatic synthesis provides an alternative to chemical synthesis. Thus, biology

laboratories may now be able to synthesize LNA comprising oligonucleotides. Other objects and advantages will be apparent from the specification.

In a first aspect, the present invention provides a method of enzymatic synthesis
5 of an oligonucleotide comprising an LNA unit, the method comprising the steps of

- a. Providing a nucleotide mix comprising an LNA nucleotide
- b. Providing a polymerase
- 10 c. Providing a template
- d. Incubating the components of steps a-c under conditions enabling synthesis of an oligonucleotide comprising an LNA unit.

15

Conditions enabling synthesis will typically be the same as those used, when LNA units are not built into the oligonucleotide. Thus, if the polymerase is a commercially available polymerase, the instructions of the supplier may be followed, optionally with adjustments. Key parameters that may be adjusted are
20 e.g. temperature, ionic strength and also the concentration of divalent cations, such as Mg^{2+} and/or Mn^{2+} . The natural nucleotide with the same base as the LNA nucleotide may be omitted from the reaction or may be present at a reduced concentration. In the latter case, a mixture of LNA units and natural units with the same base will be incorporated. The concentration of the LNA nucleotide may be the
25 same as the concentration of the other individual natural nucleotides. Preferably, the concentration is increased relatively to the concentration of the natural nucleotides. An optimal concentration of the LNA nucleotide may be determined for a particular polymerase by monitoring product yield (i.e. yield of the LNA comprising oligonucleotide) vs. the concentration of LNA nucleotide employed in
30 the reaction. Such tests can easily be set up by a man skilled in the art. In a similar way, the optimal reaction temperature, ionic strength etc. can be determined.

The term oligonucleotide as used herein does not imply any limitations as to
35 number of monomers.

Thus, the oligonucleotide comprising LNA may be of any length. Preferred lengths are between 10 and 10.000 units, more preferably the length is between 30 and 5000 units and most preferably, the length is between 50 and 1000 units.

5

In another preferred embodiment, the number of monomers (also referred to as units) in the oligonucleotide is selected from the group consisting of more than 2, more than 4, more than 6, more than 8, more than 10, more than 12, more than 15, more than 20, more than 30, more than 40, more than 60 and more than

10 100.

In another preferred embodiment, the number of monomers in the oligonucleotide is selected from the group consisting of less than 4, less than 6, less than 8, less than 10, less than 12, less than 15, less than 20, less than 30, less than 40, less than 60, less than 100, less than 200 and less than 500.

If the oligonucleotide is synthesized by extending a primer, the monomers of the primer is not taken into account, when referring the above lengths.

20 A unit as used herein refers to an RNA monomer, a DNA monomer or an LNA monomer. An RNA monomer, a DNA monomer or an LNA monomer may also be referred to as respectively an RNA nucleotide, a DNA nucleotide and an LNA nucleotide. When the nucleotide is part of an oligonucleotide, the nucleotide is a nucleotide monophosphate. When referring to nucleotides that are to be

25 enzymatically incorporated, reference is made to nucleotide triphosphates.

When referring to a "base", what is meant herein is the nucleobase of a nucleotide or nucleoside. Thus, the base may be part of e.g. an RNA monomer, a DNA monomer or an LNA monomer.

30

In a preferred embodiment, the template is a single stranded nucleic acid and the method further comprises

e. Providing a primer

35

- f. Incubating the nucleotide mix, the polymerase, the template and the primer under conditions enabling enzymatic extension of the primer, such that the primer becomes part of the LNA comprising oligonucleotide.

5

A primer as used herein is a single stranded oligonucleotide that can be extended by a polymerase. Preferred primers are between 10-30 units in length, even more preferably between 15 and 25 units. The primer is typically DNA, although RNA or LNA units may also be present in the primer. LNA units may be included for
10 stronger hybridization to the template, and thus to enable the use of short primers e.g. primers of a length even below 10 units, or to improve the biostability of the primer.

A single stranded nucleic acid as used herein is essentially the same as an
15 oligonucleotide. However, the template may be of natural origin e.g. purified from a bacterial sample or a sample of human origin such as a blood sample.

In one preferred embodiment, the nucleotide mix comprises deoxynucleotides. The nucleotide mix may comprise one, two, three or four different
20 deoxynucleotides and their individual concentration may vary. Thus, deoxynucleotides with the same base(s) as the included LNA nucleotides may be omitted or reduced in concentration. In another embodiment, the concentration of LNA nucleotides is reduced in concentration compared to deoxynucleotides of the same base. In this way, it may e.g. be possible to incorporate 1 LNA nucleotide
25 for every 5 deoxynucleotides of the same base.

Preferably, the concentration of the LNA nucleotide is between 10 mM and 150 μ M and normally, a concentration between 150 μ M and 750 μ M is used.

30 In a preferred embodiment, the nucleotide mix comprises 2 deoxynucleotides selected from the group consisting of dATP, dGTP, dCTP and dTTP. In other preferred embodiments, the nucleotide comprises 3 or 4 deoxynucleotides selected from the group consisting of dATP, dGTP, dCTP and dTTP.

The nucleotide mix may comprise an LNA nucleotide selected from the group consisting of LNA-ATP, LNA-TTP, LNA-GTP and LNA-CTP. In other embodiments, the nucleotide comprises 2, 3, or 4 nucleotides selected from the group consisting of LNA-ATP, LNA-TTP, LNA-GTP and LNA-CTP.

5

In a preferred embodiment, the nucleotide mix comprises LNA-ATP and LNA-TTP.

The above considerations regarding concentrations and composition of the nucleotide mix also apply when the nucleotide mix comprises ribonucleotides for
10 synthesis of RNA as further outlined below. Thus, deoxynucleotides in the above embodiments may be substituted for ribonucleotides for synthesis of RNA.

The template may be DNA or RNA. In one embodiment, the template may also comprise non-natural units such as LNA units. This can be the case when the
15 template has been synthesized by the method of the invention, e.g. as part of a PCR reaction.

When a primer is used, the polymerase is typically a primer dependent DNA polymerase. The polymerase may be DNA-directed or RNA directed. In the latter
20 case, the reaction is a so-called reverse transcription. Polymerases capable of reverse transcription are typically provided from retrovirus and examples are AMV reverse transcriptase, HIV-1 reverse transcriptase and M-MLV reverse transcriptase.

25 Preferred DNA polymerases are selected from the group consisting of phusion high fidelity polymerase, therminator polymerase, KOD DNA polymerase, and 9 ° north polymerase. In one embodiment, it is preferred to use a polymerase deficient in proofreading. In another embodiment, it is preferred that the polymerase is capable of proof reading.

30

9 ° north polymerase has been described by Southworth et al, 1996.

Therminator DNA polymerase, is an A485L point mutant of the 9°N DNA polymerase.

35

Phusion high fidelity polymerase is an enzyme developed and manufactured by Finnzymes Oy and distributed by New England Biolabs Inc. Incorporating a new fusion protein technology developed by Finnzymes Oy in collaboration with MJ Bioworks, Inc, Phusion High-Fidelity DNA Polymerase brings together a novel
5 Pyrococcus-like enzyme with a processivity-enhancing domain. By fusing a double-strand DNA binding domain to the polymerase, its processivity can easily be increased 10-fold.

KOD DNA polymerase (a recombinant form of *Thermococcus kodakaraensis* KOD1 DNA polymerase) can be obtained from Novagen and has been described by Nishoka et al., 2001.

- Preferably, the LNA comprising oligonucleotide comprises at least 2 LNA units.
10 Thus, in a preferred embodiment, the oligonucleotide comprises a number of LNA units selected from the group consisting of more than 1 LNA unit, more than 2 LNA units, more than 3 LNA units, more than 5 LNA units, more than 7 LNA units, more than 10 LNA units, more than 15 LNA units and more than 20 LNA units.
- 15 The LNA units may be identical or comprise different bases.

The LNA nucleotides (and corresponding LNA units of the LNA comprising oligonucleotide) employed in the method of the invention is preferably selected from the group consisting of LNA, amino LNA (also called 2'-amino-LNA), 2'-N-
20 acetyl-2'-amino-LNA (also called N-acetyl amino LNA), other N2'-acylated derivatives of 2'-amino-LNA nucleotides, N2'-alkylated derivatives of 2'-amino-LNA nucleotides, 2'-thio-LNA nucleotides and base-modified LNA nucleotides like 5-substituted pyrimidine LNA nucleotides.

- 25 In one embodiment, amino LNA and N-acetyl amino LNA is preferred.

In a preferred embodiment, the method of the invention further comprises the steps of

- 30 g. Separating the LNA comprising oligonucleotide from the template by denaturation

- h. Hybridizing a second primer to the LNA comprising oligonucleotide
- i. Extending said second primer using enzymatic synthesis

5

Thus, performing steps g-i will synthesize a new copy of the template that was provided in step c.

As will be apparent, the steps can be further repeated, whereby more copies of
10 the template and first strand (the strand complementary to the template) are synthesized in a process that is essentially PCR, but with incorporation of LNA units in the PCR product.

Separation of the LNA comprising oligonucleotide from the template is preferably
15 done by denaturation, which in turn may be done by e.g. increasing the pH, lowering the ionic strength or more feasibly by increasing the temperature of the sample.

If the employed polymerase does not tolerate the conditions used for
20 denaturation, a new supply of polymerase should be added after denaturation. Most preferably, a heat stable polymerase is used, such that denaturation can be performed using a temperature increase to e.g. 94 °C. Such polymerases are typically used for PCR and a variety exists.

25 In a preferred embodiment, at least 5 repetitions are performed. Even more preferably, between 20 and 40 repetitions are performed.

In one embodiment, the template is a double stranded nucleic acid comprising a transcriptional promoter enabling RNA synthesis. The transcriptional promoter is
30 preferably selected from the group consisting of SP6 polymerase, T7 polymerase and T3 polymerase.

Thus, the reaction will be recognized as in vitro transcription with incorporation of LNA units into the resulting transcript (LNA comprising RNA oligonucleotide).

35

As mentioned earlier, ribonucleotides are used for synthesis of RNA and the above considerations for deoxynucleotides also apply for ribonucleotides.

The double stranded nucleic acid may be a plasmid or a PCR product. When the
5 double stranded nucleic is a plasmid, the plasmid will typically be linearized to enable production of run-off transcripts.

In vitro transcription of transcripts comprising LNA units are of interest for several reason, e.g. because the biostability of the resulting transcript is improved.

10

This is very important when the in vitro transcription reaction is part of a so-called SELEX process. SELEX is a process where a large pool of nucleic acids, e.g. 10^{14} different RNA transcripts, is repeatedly fractionated against a target and re-synthesized. In this way, identification of high affinity RNA transcripts is possible.
15 The RNA transcripts are often termed aptamers, because they bind their target as a result of their three dimensional structure. Unfortunately, aptamers identified by SELEX most often have a poor biostability, which has to be improved by post-SELEX modifications. The method of the invention may be used to directly identify LNA comprising aptamers that have an improved biostability as compared to a
20 corresponding aptamer without LNA. Moreover, incorporation of LNA stabilizes secondary structures (base pairing) which may in turn give an overall more rigid three-dimensional structure.

Furthermore, because of the affinity-enhancing effect of LNA units, the selection
25 or development of relatively short LNA-comprising aptamers is enabled, i.e. aptamers that contain segments that are formed by intramolecular base pairing that would not have been formed in a non-LNA comprising aptamer or construct.

30 Importantly, SELEX may also be performed using a pool of DNA oligonucleotides, in which case one of the strands of the PCR product is selected against a target.

For SELEX, the method preferably comprises a plurality of templates. The templates may be synthesized using standard methods for oligonucleotide

synthesis. In a preferred embodiment, the templates comprise LNA units. In another embodiment, the templates do not comprise any LNA units.

The plurality of templates may be obtained by performing a selection process. A
5 preferred selection process comprises fractionating the plurality of templates for affinity toward a target to enrich for templates that binds to the target.

In another embodiment, the oligonucleotide comprising an LNA unit is selected against a target. As will be understood, when a plurality of templates are used for
10 enzymatic synthesis, a plurality of different oligonucleotides comprising an LNA unit will be synthesized. Regardless of when a selection process is performed, an enrichment of sequences that bind to the target should be achieved.

If many rounds of selection (enrichment for binders) and enzymatic synthesis
15 (amplification) are performed, the pool of oligonucleotides will eventually comprises a limited number of sequences that can be individually identified. E.g. by cloning and sequencing of oligonucleotides. Therefore, the selection process enriching for templates and/or oligonucleotide comprising an LNA unit that binds to a target and enzymatic synthesis of an oligonucleotide comprising an LNA unit
20 is preferably repeated until oligonucleotides binding to the target with high affinity can be identified. Preferably 8 rounds of selection and amplification are performed.

The number of different sequences in the template is preferably at least 10^{14} . In
25 another embodiment, the number is at least 10^{12} .

Preferably, the target is a protein, e.g. of human origin.

30 A second aspect of the invention is a kit comprising a polymerase, a nucleotide mix and a LNA nucleotide.

The kit may be adapted to perform any of the embodiments of the method of the invention and hence comprise the appropriate set of components. I.e.
35 ribonucleotides if the polymerase is a RNA polymerase and deoxynucleotides if the

polymerase is a DNA polymerase. In a preferred embodiment, the nucleotide mix and the LNA nucleotide is present in the same tube in optimized concentrations for the particular polymerase and application.

5 Further, the kit may include instructions for use.

A third aspect of the invention is the use of an LNA nucleotide for enzymatic DNA synthesis, for enzymatic RNA synthesis or for use in PCR, as described in the first aspect of the invention.

10

A fourth aspect of the invention is use of a polymerase for the preparation of an oligonucleotide comprising an LNA unit, as described in the first aspect of the invention.

15 A fifth aspect of the invention is use of a LNA nucleotide for SELEX.

Examples

The following examples demonstrate incorporation of LNA-nucleotides using
20 various polymerases and LNA-triphosphates as reagents. In some experiments, the efficiency of the reaction is improved when using LNA nucleotides. In others, the product of the reaction has an improved stability under the conditions employed, i.e. the LNA units protect the product from being degraded.

25 Also PCR amplification using various polymerases and LNA-triphosphates is demonstrated.

Materials and Methods

The DNA primer sequences were purchased from DNA Technology and the
30 template sequences from Sigma-Genosys. Phusion™ High Fidelity DNA Polymerase was purchased from Finnzymes and 9 °N DNA polymerases was purchased from New England Biolabs. KOD DNA polymerase was obtained from Novagen.

All experiments included positive control and negative control reactions in parallel with the reactions involving incorporation of LNA monomers. The positive control reaction mixture consisted of all four natural dNTPs and hence resulted in extension to full length. The negative control reaction mixture lacked the
5 nucleoside triphosphates similar to the LNA nucleotides that were to be tested for enzymatic incorporation, and thus contained three or two natural nucleoside triphosphates. Thus, the negative control experiments were expected to result in extension only up to the nucleotide before the first site of LNA incorporation. In all experiments, the reaction mixture was supplemented with MnCl_2 as Mn^{2+} is known
10 to increase the tolerability of polymerases with respect to incorporation of nucleotides having modifications in the furanose ring, the base moiety or the phosphate linkage. Betaine enhancer solution was also added as it is known to be an effective additive for templates which are difficult to amplify.

15 *Synthesis of LNA 5'-triphosphates:* LNA nucleoside 5'-triphosphates were synthesised according to the one-pot synthesis method described by Ludwig et al, 1981. After the addition of tributyl-ammonium pyrophosphate, the reaction mixture was stirred for 2.5 h before the reaction was quenched by the addition of triethyl ammonium bicarbonate. Purification and isolation involved gravity
20 chromatography of the crude product on WHATMAN DEAE cellulose- D50 anion-exchange resin by eluting with an increasing concentration of triethyl ammonium bicarbonate in water.

General procedure for primer extension assays: The primer sequences were 5'-³²P
25 labelled by reaction with [γ -³²P]-ATP (~6000 Ci/mmol, Amersham Biosciences) using T4 polynucleotide kinase (New England Biolabs) following manufactures recommendations. The 5'-end labelled primers were annealed to the templates by combining primer and template in a molar ratio of 1:1 and heating the mixture to 80 °C for 2 min, followed by slow cooling to room temperature. The reaction
30 mixtures were prepared in a total volume of 20 μL by adding 0.6 μL of a solution containing 5'-³²P labelled primer-template (5 pmol) complex, 4 μL of 5 \times Phusion HF buffer (included in the PhusionTM High Fidelity DNA Polymerase Kit, Finnzymes), 1 μL of MnCl_2 (50 mM), 1 μL of betaine enhancer solution (2 M, Ampliqon), 1 μL of dNTP mixture (10 mM in each dNTP; for the preparation of
35 nucleoside triphosphate mixtures containing LNA 5'-triphosphates, 100 mM of LNA

triphosphates were used), 1 μL of PhusionTM High Fidelity DNA Polymerase (2U/ μL) and 11.6 μL of distilled water (two times distilled). The reaction mixtures were gently vortexed and heated at 72 $^{\circ}\text{C}$ for 2 h. The polymerase reactions were quenched by the addition of a double volume of loading buffer (95% formamide, 5 0.05% bromophenol blue, 0.05% xylene cyanol and 20 mM EDTA). Analysis was performed by gel electrophoresis for 30 min on a 13% 7 M urea polyacrylamide gel in the presence of a TBE buffer (100 mM Tris, 90 mM Boric acid, 1 mM EDTA) of pH 8.4. Products were visualized by phosphor imaging.

10 *General procedure for polymerase chain reaction:* Both forward and reverse primer sequences were 5'-³²P labelled by reaction with [γ -³²P]-ATP (~6000 Ci/mmol, Amersham Biosciences) using T4 polynucleotide kinase (New England Biolabs) following manufactures recommendations. The PCR reaction mixtures were prepared in a total volume of 20 μL by adding 0.35 μL each of a solution
15 containing 5'-³²P labelled primers (50 pmol), 0.25 μL template (0.5 μM), 4 μL of 5 \times polymerase buffer (included in the DNA Polymerase Kit,), 1 μL of MnCl_2 (50 mM), 1 μL of betaine enhancer solution (2 M, Ampliqon), 1 μL of dNTP mixture (10 mM in each dNTP; for the preparation of nucleoside triphosphate mixtures containing LNA 5'-triphosphates, 10 mM of LNA triphosphates were used), 1 μL of
20 DNA Polymerase (2U/ μL) and 12.7 μL of distilled water (two times distilled). The reaction mixtures were gently vortexed and amplified using a MastercyclerTM (Eppendorf) at desired cycling conditions. The polymerase reactions were quenched by the addition of a double volume of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 20 mM EDTA). Analysis was
25 performed by gel electrophoresis for 30 min on a 13% 7 M urea polyacrylamide gel in the presence of a TBE buffer (100 mM Tris, 90 mM Boric acid, 1 mM EDTA) of pH 8.4. Products were visualized by phosphor imaging.

Example 1

30 *Pfu* DNA polymerase stops extension after incorporation after the first LNA nucleotide has been incorporated.

LNA-T has been investigated as substrate of *Pfu* DNA polymerase using 19n long primer and 44n long DNA template having three sites of incorporations at positions 37, 39 and 41. It was observed that *Pfu* DNA polymerase can extend the
35 primer only up to 37 by incorporating one LNA-T nucleotide and stopped further

extension. Whereas we observed control reactions using natural nucleotides yielded the products at expected length.

The results are shown in figure 1.

5 Example 2

Phusion high fidelity DNA polymerase enables incorporation of multiple LNA-T units.

The incorporation of LNA-T nucleotide was performed using a 44n long DNA template having three sites of incorporations at positions 37, 39 and 41. Phusion
10 high fidelity DNA polymerase efficiently incorporated the LNA-T nucleotides and extended the 19n long primer to full length in comparable yields to those of the natural nucleotide experiments conducted in parallel as a control.

The results are shown in figure 2.

15 Example 3

Phusion high fidelity DNA polymerase enables incorporation of multiple LNA-A units.

To study incorporation of LNA-A monomer, a 42n long DNA template was designed which contains two incorporation sites at positions 38 and 41. The
20 experiments showed that Phusion™ High Fidelity DNA Polymerase efficiently extended the primers to full length after incorporating the LNA-A nucleotides
The results are shown in figure 3.

Example 4

25 Phusion high fidelity DNA polymerase enables incorporation of multiple LNA-A and LNA-T units.

For incorporation of both LNA-T and LNA-A nucleotides into one DNA strand, A 43n long DNA template was designed to enforce incorporation of first three LNA-T monomers and then three LNA-A monomers. Primer extension to full length was
30 again observed demonstrating the capability of the enzyme to incorporate two different LNA modified nucleotides in one reaction leading to a substantially modified LNA-modified DNA strand.

The results are shown in figure 4.

Example 5

Phusion high fidelity DNA polymerase enables incorporation of successive LNA-A and LNA-T units.

Multiple and successive incorporation of LNA-T and LNA-A nucleotides was likewise
5 attempted using Phusion™ High Fidelity DNA Polymerase. For these experiments
we designed template DNA strands containing segments encoding for
incorporation of eight continuous LNA nucleotides in the primer strand. These
experiments showed that the enzyme is able to incorporate up to three LNA-T
nucleotides successively. However, in the case of LNA-A nucleotide, all eight
10 modified nucleotides were incorporated, whereupon no further extension was
observed.

The results are shown in figures 5 and 6.

Example 6

15 LNA-TTP has rendered satisfactory fidelity compared to dTTP

Although we observed a partial extension to one nucleotide more from the
expected stop, elongation to full length was not observed for the 44n long
template in the presence of only LNA-T triphosphate, dCTP and dGTP. As arrest of
chain extension was observed at the expected positions, this is a first indication of
20 satisfactory fidelity of LNA-T incorporation.

The results are shown in figure 7.

Example 7

LNA-A has similar fidelity as compared to dATP.

25 Elongation to full length was again not observed for the 43n long template in the
presence of only LNA-A triphosphate, dCTP and dGTP. As arrest of chain extension
was observed at the expected positions, this is a first indication of satisfactory
fidelity of LNA-A incorporation.

The results are shown in figure 8.

30

Example 8

Phusion high fidelity DNA polymerase enables incorporation of several amino-LNA-TTP (at most 2).

The results are shown in figure 9.

Example 9

Phusion high fidelity DNA polymerase enables incorporation of multiple LNA

5 nucleotides on a LNA modified template.

Incorporation of both LNA-T and LNA-A nucleotides was performed using a 43n long LNA modified (at positions 31, 33 for LNA-T nucleotides and positions 36 and 39 for LNA-A nucleotides) template DNA strand. Primer extension to full length was again observed demonstrating the capability of the enzyme to incorporate

10 two different LNA modified nucleotides opposite to the LNA-modified nucleotides in the template strand.

The results are shown in figure 10.

Example 10

15 Terminator polymerase enables incorporation of multiple LNA-A nucleotides.

The results are shown in figure 11.

Example 11

Comparison of LNA-T incorporation by phusion high fidelity polymerase and 9 °

20 north polymerase.

At 65 ° C, phusion polymerase is more active using natural nucleotides. At 72 ° C, the efficiency is approximately the same. 9 ° North polymerase has a comparable activity with regards to incorporation of LNA nucleotides and natural nucleotides at both temperatures. However, LNA seems to prevent degradation of the

25 extension product at 72 °, i.e. LA confers stability on the product.

The results are shown in figure 12.

Example 12

9 ° North polymerase enables incorporation of multiple LNA-A nucleotides.

30 The results are shown in figure 13.

Example 13

9 ° North polymerase enables incorporation of multiple LNA-A and LNA-TTP with an improved efficiency as compared to incorporation of natural nucleotides.

The results are shown in figure 14.

5

Example 14

Phusion polymerase enables PCR amplification using LNA-A.

The conditions and results are shown in figures 15-19.

10 Example 15

Phusion polymerase enables PCR amplification using LNA-T with an improved efficiency as compared to using the dT.

The conditions and results are shown in figures 20-21.

15 Example 16

9 ° North polymerase enables highly efficient PCR.

The result indicates that incorporation of LNA nucleotides protects the PCR product from degradation, as the product of the positive control (lane 3, marked +) seems to be partly degraded.

20 The results are shown in figures 22-23.

Example 17

PCR using LNA-ATP using 9° N DNA polymerase.

9 ° North polymerase enables highly efficient PCR.

25 The result indicates that incorporation of LNA nucleotides protects the PCR product from degradation, as the product of the positive control (lane 3, marked +) seem be partly degraded.

The results are shown in figure 24.

30 Example 18

Use of LNA nucleotides improves the efficiency of PCR using 9 ° North polymerase.

The results are shown in figure 25.

Example 19

PCR amplification using LNA-A and LNA-T.

Under the conditions of the present experiment, incorporation was not efficient.

5 However, also positive controls did not work very well (not shown).

The results are shown in figure 26.

Example 20

PCR without annealing step.

10 The result shows that PCR without an annealing step did not progress efficiently.

The results are shown in figure 27.

Example 21

Phusion polymerase enables incorporation of N-methyl amino LNA-T.

15 However, the polymerase does not extend beyond incorporation of the first LNA-T.

The results are shown in figure 28.

Example 22

9 ° North polymerase enables incorporation of N-methyl amino LNA-T.

20 The polymerase efficiently incorporated one LNA-TTP to produce a stable product.

Very little product is observed for the positive control, which interpreted as degradation of the product. A faint full length product is visible in the positive control.

The results are shown in figure 29.

25

Example 23

Phusion polymerase is inefficient in incorporating N-methylamino LNA-T.

We have performed the experiment to incorporate N-methylamino LNA-T using a 43n long template and the result showed no incorporation of LNA building block.

30 The results are shown in figure 30.

Example 24

Phusion polymerase is enables efficient incorporation of N-acetyl amino LNA-T.

The results are shown in figure 31.

Example 25

9 ° North polymerase enables efficient incorporation of N-acetyl amino LNA-T. The efficiency is improved as compared to using natural nucleotides. Again this result is interpreted to be caused by degradation of the product of the positive control. The results are shown in figure 32.

Example 26

10 Phusion polymerase enables efficient incorporation of amino LNA-T. Again the positive control seems to be degraded. The results are shown in figure 33.

Example 27

15 9 ° North polymerase enables incorporation of amino LNA-T. The results are shown in figure 34.

Example 28

Phusion polymerase enables efficient incorporation of amino LNA-T. The efficiency is improved as compared to using natural nucleotides. The results are shown in figure 35.

Example 29

9 ° North polymerase enables incorporation of amino LNA-T. The efficiency is improved as compared to using natural nucleotides. Again the positive control may be degraded. The results are shown in figure 36.

Example 30

30 The efficiency of LNA-T incorporation by Phusion polymerase improves by increasing the reaction time. The results are shown in figure 37.

Example 31

The efficiency of LNA-T incorporation by 9 ° North polymerase is not significantly affected by the reaction time. However, prolonged reaction time decreases the amount of product, when natural nucleotides are used indication degradation of
5 the PCR product.

The results are shown in figure 38.

Example 32

Phusion polymerase is inefficient with regards to incorporation of N-acetyl amino
10 LNA-T using a different template.

The results are shown in figure 39.

Example 33

9 ° North polymerase is inefficient regards to incorporation of N-acetyl amino
15 LNA-T using a different template.

The results are shown in figure 40.

Example 34

Amino LNA-TTP has rendered satisfactory fidelity compared to dTTP using Phusion
20 polymerase

Although we observed a partial extension to one nucleotide more from the expected stop, elongation to full length was not observed for the 44n long template in the presence of only aminoLNA-T triphosphate, dCTP and dGTP. As arrest of chain extension was observed at the expected positions, this is a first
25 indication of satisfactory fidelity of amio LNA-T incorporation.

The results are shown in figure 41.

Example 35

Phusion polymerase enables incorporation of LNA-T on LNA modified template
30 Incorporation of amino LNA-T nucleotides was performed using a 43n long LNA modified (at positions 31, 33 for amino LNA-T nucleotides and positions 36 and 39 for dA nucleotides) template DNA strand. Primer extension to full length was again observed demonstrating the capability of the enzyme to incorporate amino LNA-T

modified nucleotides opposite to the LNA-A modified nucleotides in the template strand.

The results are shown in figure 42.

5 Example 36

Phusion polymerase enables incorporation of LNA-T and LNA-A

Incorporation of both amino LNA-T and LNA-A nucleotides was performed using a 43n long LNA modified (at positions 31, 33 for amino LNA-T nucleotides and positions 36 and 39 for LNA-A nucleotides) template DNA strand. Primer extension
10 to full length was again observed demonstrating the capability of the enzyme to incorporate two different LNA modified nucleotides opposite to the LNA-modified nucleotides in the template strand.

The results are shown in figure 43.

15

Example 37

Incorporation of LNA-A using T7 RNA polymerase:

20 Primer 5'-TAATACGACTCACTATAGG-3', 19n

Template 3'-ATTATGCTGAGTGATATCCGACCGGCGACGCGCCAGGTACTG-5', 42n

Product 5'-GGCUGGCCGCGUGCGCGGUCCAUGAC-3' (25n transcript)

25

Procedure :

Annealing

30

Hybridisation buffer, 5	4.0 μ L
Primer, 100 pmol/ μ L	0.4 μ L
Template, 100	0.4 μ L
H ₂ O, dd	15.2
Total	20 μ L

The reaction mixture was incubated at 80 °C for 3 min followed by slow cooling to 37 °C

5 Transcription Reaction:

Annealing mix	5.00 µL
TSC buffer, 20 x	2.50 µL
GTP, 100 mM	1.50 µL
UTP, 100 mM	1.50 µL
ATP/LNA-ATP, 100 mM	1.50 µL
CTP+[α-32P]-CTP, 100 mM	1.50 µL
PEG 8000, 400 mg/mL	6.25 µL
MgCl ₂ , 1 M	0.70 µL
Sodium glutamate, 1 M	1.25 µL
H ₂ O,dd	2.05 µL
T7 RNA polymerase	1.25 µL
Total	25 µL

The reaction mixture was incubated at 37 °C overnight

10

Result:

The results showed that full length transcripts (25n long RNA) were obtained in both LNA-A reaction (Figure 44, lane 1) and positive control reaction using all four natural rNTPs (Figure 44, lane 2). Thus, LNA-A can be incorporated using T7 RNA

15 polymerase.

Example 38

KOD DNA polymerase enables incorporation of multiple LNA-A, LNA-T and LNA-5-methyl C nucleotides.

The incorporation was performed using a 40n long DNA template having five sites
20 of incorporations for LNA-A, four sites of incorporations for LNA-5-methyl C and three sites of incorporations for LNA-T nucleotides. KOD DNA polymerase

efficiently incorporated all of the three LNA nucleotides and extended the 19n long primer to full length.

The results are shown in figure 45.

5 Example 39

KOD DNA polymerase enables incorporation of successive multiple LNA-5-methyl C nucleotides.

The incorporation was performed using a 44n long DNA template having a segment of eight consecutive incorporation sites for LNA-5-methyl C nucleotides.

- 10 KOD DNA polymerase efficiently incorporated all of the eight LNA-5-methyl C nucleotides and extended the 19n long primer to full length.

The results are shown in figure 46.

Example 40

- 15 KOD DNA polymerase enables incorporation of multiple LNA-A, LNA-T and LNA-5-methyl C nucleotides from an LNA nucleotides only mixture.

The incorporation was performed using a 34n long DNA template having eight sites of incorporations for LNA-A, four sites of incorporations for LNA-5-methyl C and three sites of incorporations for LNA-T nucleotides. KOD DNA polymerase

- 20 extended the primer up to six nucleotides by incorporating three LNA-A, one LNA-T and one LNA-5-methyl C nucleotides.

The results are shown in figure 47.

Example 41

- 25 PCR using LNA-ATP using KOD DNA polymerase.

KOD polymerase enables a highly efficient PCR.

The result indicates that an efficient amplification of LNA-containing DNA strand in an excellent yield compared to those of natural nucleotide experiment at the specified reaction conditions.

- 30 The conditions and results are shown in figure 48 and 49.

Example 42

Verification of the PCR product (Example 42) by MALDI-TOF MS analysis.

MALDI-TOF MS analysis of the PCR products indicated that the observed masses are in excellent agreement with that of the theoretical masses which clearly show the fidelity of LNA-A incorporations by KOD DNA polymerase.

The results are shown in figure 50.

5

Example 43

Incorporation of LNA-U using T7 RNA polymerase:

Similar experimental procedures were employed as for example 37. The results showed that full length transcripts (26n long RNA) were obtained in both LNA-A
10 reaction (Figure 51). Thus, LNA-U can be incorporated using T7 RNA polymerase.

15

20

25

30

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Claims:

1. A method of enzymatic synthesis of an oligonucleotide comprising an LNA unit, the method comprising the steps of
 - a. Providing a nucleotide mix comprising an LNA nucleotide
 - b. Providing a polymerase
 - c. Providing a template
 - d. Incubating the components of steps a-c under conditions enabling synthesis of an oligonucleotide comprising an LNA unit.
2. The method of claim 1, wherein the template is a single stranded nucleic acid and wherein the method further comprises
 - a. Providing a primer
 - b. Incubating the nucleotide mix, the polymerase, the template and the primer under conditions enabling enzymatic extension of the primer, such that the primer becomes part of the LNA comprising oligonucleotide.
3. The method of any of the preceding claims, wherein the nucleotide mix comprises dATP, dGTP, dCTP and dTTP.
4. The method of any of the preceding claims, wherein the template is DNA or RNA.
5. The method of any of the preceding claims, wherein the polymerase is a DNA polymerase selected from the group consisting of phusion high fidelity polymerase, therminator polymerase, 9^o north polymerase and KOD DNA polymerase

6. The method of any of the preceding claims, wherein the nucleotide mix comprises LNA-A and/or LNA-T.
- 5 7. The method of any of the preceding claims, wherein the nucleotide mix comprises LNA-A, LNA-T, and LNA-5-methyl-C together with or without natural nucleotides.
- 10 8. The method of any of the preceding claims, wherein the LNA comprising oligonucleotide comprises at least 2 LNA units, such as 3, 4, or 5 units.
- 15 9. The method of any of the preceding claims, wherein the LNA nucleotide is selected from the group consisting of LNA, amino LNA (also called 2'-amino-LNA), 2'-N-acetyl-2'-amino-LNA (also called N-acetyl amino LNA), other N2'-acylated derivatives of 2'-amino-LNA nucleotides, N2'-alkylated derivatives of 2'-amino-LNA nucleotides, 2'-thio-LNA nucleotides and base-modified LNA nucleotides like 5-substituted pyrimidine LNA nucleotides.
- 20 10. The method of any of the preceding claims further comprising the steps
 - a. Denaturing the LNA comprising oligonucleotide hybridized to the template
 - 25 b. Hybridizing a second primer to the LNA comprising oligonucleotide
 - c. Extending said second primer using enzymatic synthesis
11. The method of claim 9, wherein at least 5 repetitions are performed.
- 30 12. The method of any of claims 1, 4, 6, 7 and 8, wherein the template is a double stranded nucleic acid comprising a transcriptional promoter enabling RNA synthesis.

13. The method of claim 11, wherein the polymerase is a RNA polymerase selected from the group consisting of SP6 polymerase, T7 polymerase and T3 polymerase.
- 5 14. The method of any of the preceding claims comprising a plurality of templates.
15. The method of any of the preceding claims, wherein the plurality of templates is obtained by performing a selection process.
- 10 16. The method of any of the preceding claims wherein the selection process comprises fractionating the plurality of templates for affinity toward a target to enrich for templates that binds to the target.
- 15 17. The method of any of the preceding claims, wherein the oligonucleotide comprising an LNA unit is selected against a target.
18. The method of any of the preceding claims, wherein the selection process enriching for templates and/or oligonucleotide comprising an LNA unit that binds to a target and enzymatic synthesis of an oligonucleotide comprising an LNA unit is repeated until oligonucleotides binding to the target with high affinity can be identified.
- 20 19. The method of any of the preceding claims, wherein the plurality of templates comprise at least 10^{14} different sequences.
- 25 20. The method of any of the preceding claims, wherein the target is a protein.
21. A kit comprising a polymerase, a nucleotide mix and a LNA nucleotide.
- 30 22. Use of a LNA nucleotide for PCR.
23. Use of a LNA nucleotide for transcription.

24. Use of a polymerase for the preparation of an oligonucleotide comprising a LNA unit.

25. Use of a LNA nucleotide for SELEX

5

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15

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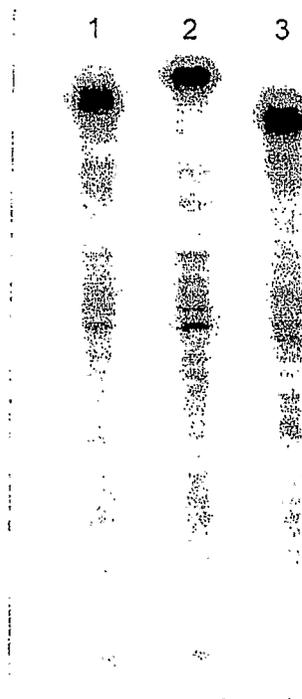
pfu DNA polymerase incorporation of LNA-T

<u>Incorporation of LNA-T</u>		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGGATAGACGG-5'	44

Fig. 1

Incorporation of II, LNA-TTP:

1. dTTP was replaced with LNA-TTP (100 mM), 37n long.
2. Positive control - using all four normal dNTP's, 44n long.
3. Negative control - dTTP or LNA-TTP was absent, 36n long.

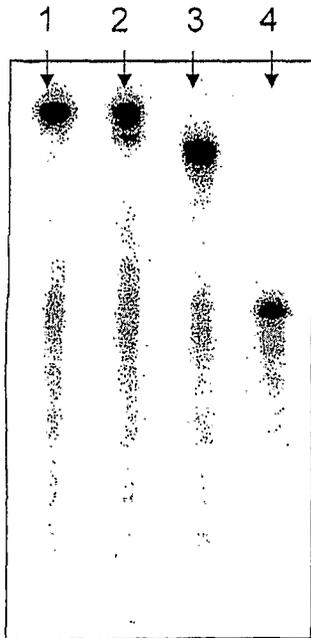


Phusion™ High Fidelity DNA polymerase incorporation of LNA-T

Incorporation of LNA-T , 1		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGG <u>A</u> T <u>A</u> G <u>A</u> CGG-5'	44

37
↓

Fig. 2

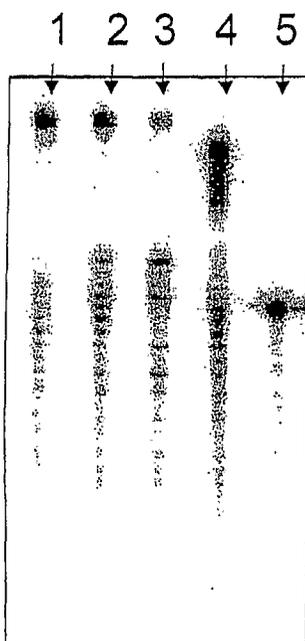


1. Positive control – using all four natural dNTPs, 44nt long
2. dTTP was replaced with LNA-TTP, 44nt long
3. Negative control – dTTP was absent in the reaction mix, 36nt long
4. Primer, 19nt long

Phusion™ High Fidelity DNA polymerase incorporation of LNA-A

Incorporation of LNA-A, 2		38	Length
Primer	5'-TAATACGACTCACTATAGG-3'	↓	19
Template	3'-ATTATGCTGAGTGATATCCGACCGGCGACGCGCCAGGTACTIG-5'		42

Fig. 3



1. Positive control – using all four natural dNTPs, 42nt long
2. dATP was replaced with LNA-ATP (300 mM), 42nt long
3. dATP was replaced with LNA-ATP (100 mM), 42nt long
4. Negative control – dATP was absent in the reaction mix, 37nt long
5. Primer, 19nt long

3/51

Phusion™ High Fidelity DNA polymerase multiple incorporation of LNA

Multiple incorporation of 1 and 2		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCG <u>ACCCACACCIGGICIGG</u> -5'	43

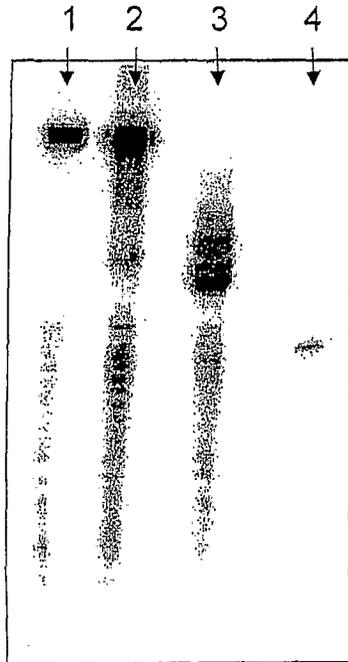


Fig. 4

1. Positive control – using all four natural dNTPs, 43nt long
2. dTTP and dATP was replaced with LNA-TTP and LNA-ATP, 43nt long
3. Negative control – dTTP and dATP was absent in the reaction mix, 26nt long
4. Primer, 19nt long

Phusion™ High Fidelity DNA polymerase successive incorporations

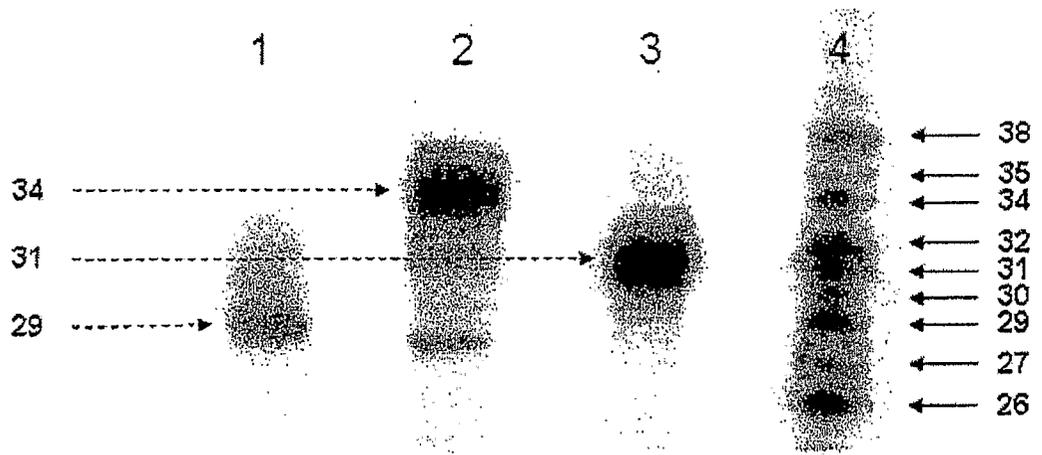
Primer and template sequences:

<u>Successive incorporation of LNA-T, 1</u>		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCCAAAAAAAAAATGGTCGCC-5'	42
	27 ↓	
<u>Successive incorporation of LNA-A, 2</u>		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCGTTTTTTTITAGACGG-5'	40
	27 ↓	
<u>Successive multiple incorporation of 1 and 2</u>		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCGTTTAATAAGCGCC-5'	39
	27 ↓	

Fig. 5

Phusion™ High Fidelity DNA polymerase successive incorporations

Fig. 6

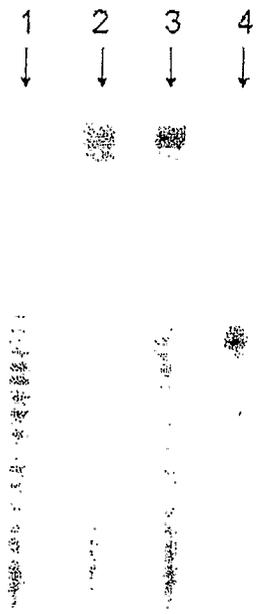
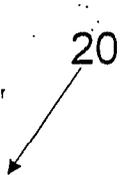


1. LNA-T nucleotide, upto 3, can be tolerated
2. LNA-A nucleotide, up to 8 can be tolerated
3. LNA-T & LNA-A together, upto 5 can be tolerated
4. ³²P end labeled DNA strands of indicated lengths

6/51

Checking the Fidelity using LNA-TTP

<u>Fidelity using LNA-TTP</u>		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGGATAGACGG-5'	44



1. Fidelity set – dCTP, dGTP and LNA-TTP only in the mix, 21-22nt.
2. dTTP was replaced with LNA-TTP, 44nt long.
3. Positive control – using all four natural dNTPs, 44nt long.
4. Primer, 19nt long.

Fig. 7

Checking the Fidelity using LNA-A

<u>Fidelity using LNA-ATP</u>			Length
Primer	5'-TAATACGACTCACTATAGG-3'	27 ↓	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCG <u>A</u> CCACACCTGGTCTGG-5'		43



1. Fidelity set – dCTP, dGTP and LNA-ATP only in the mix, 27nt
2. Positive control – using all four natural dNTPs, 43nt long
3. dATP was replaced with LNA-ATP, 43nt long
4. Primer, 19nt long

Fig. 8

Phusion™ High Fidelity DNA polymerase incorporation of Amino-LNA-T

Incorporation of Amino-LNA-T, 1			Length
Primer	5'-TAATACGACTCACTATAGG-3'	37	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGGATAGACGG-5'	↓	44

Fig. 9



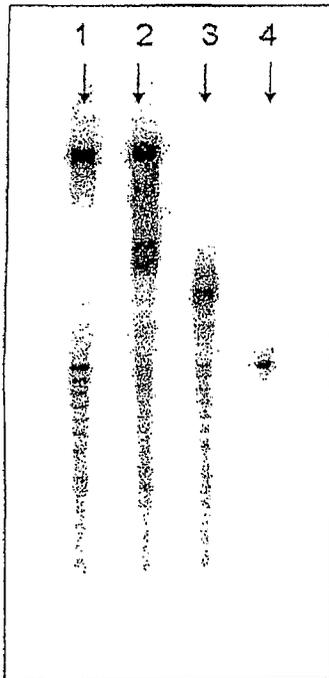
1. Positive control – using all four natural dNTPs, 44nt long
2. dTTP was replaced with Amino-LNA-TTP, 37 – 39 nt long
3. Negative control – dTTP was absent in the reaction mix, 36nt long
4. Primer, 19nt long

9/51

Phusion™ High Fidelity DNA polymerase multiple incorporation of 1 & 2 on LNA modified template

Multiple incorporation of 1 and 2		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCGACCC <u>ACACCTGGT</u> CTGG-5'	43

27
↓



1. Positive control – using all four natural dNTPs, 43nt long
2. dTTP and dATP was replaced with LNA-TTP and LNA-ATP, 43nt long
3. Negative control – dTTP and dATP was absent in the reaction mix, 26nt long
4. Primer, 19nt long

Fig. 10

Incorporation of LNA-A by Therminator™ Polymerase

Primer	5'-TAATACGACTCACTATAGGCC-3'	21
Template	3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'	43



Fig. 11

1. Positive control – using all four natural dNTPs
2. dATP was replaced with LNA-ATP, 43nt long
3. Negative control – dATP was absent in the reaction mix

Incorporation of LNA-T, a comparison of two polymerases

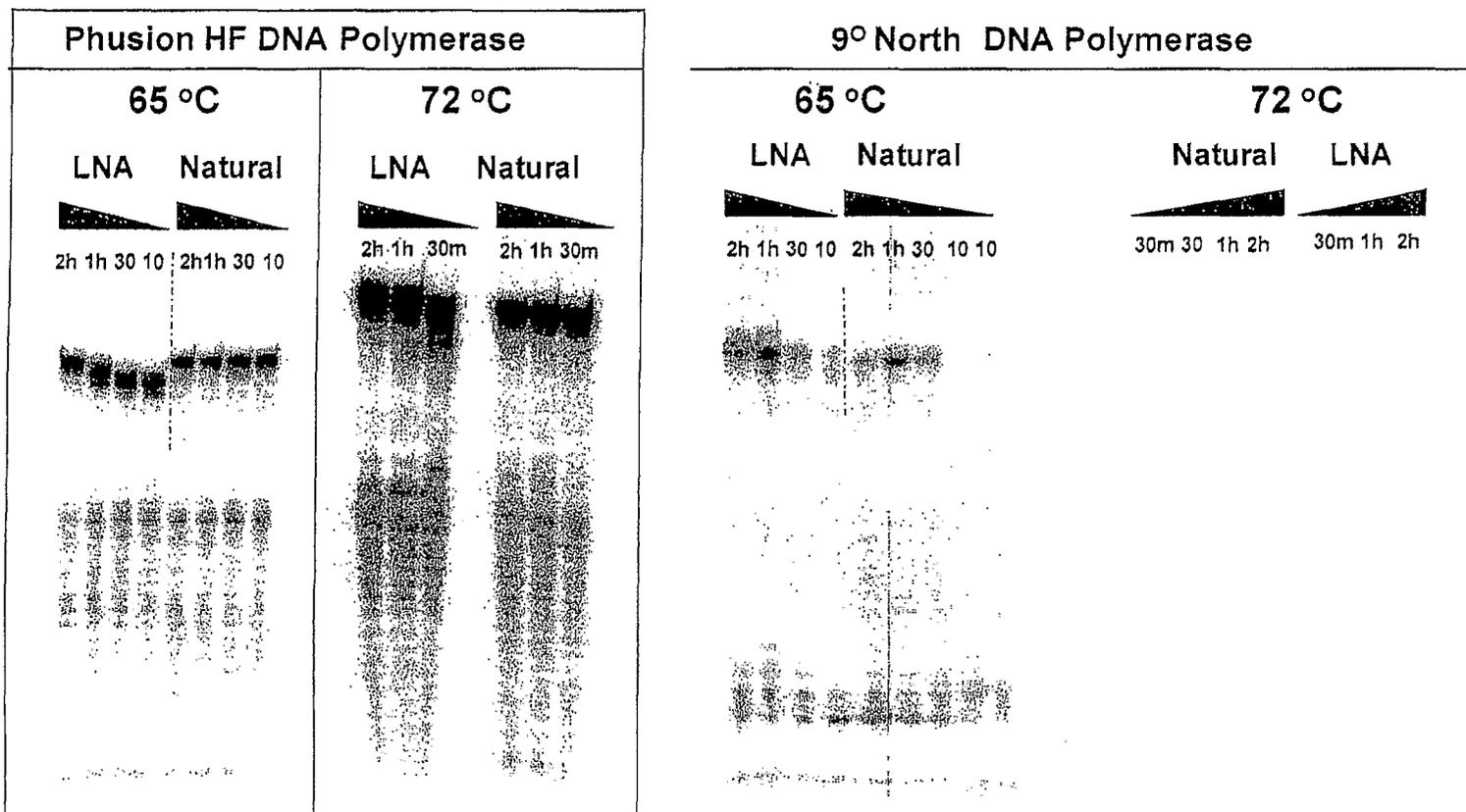
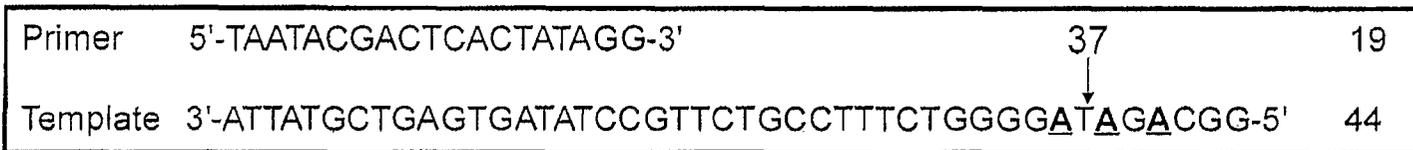
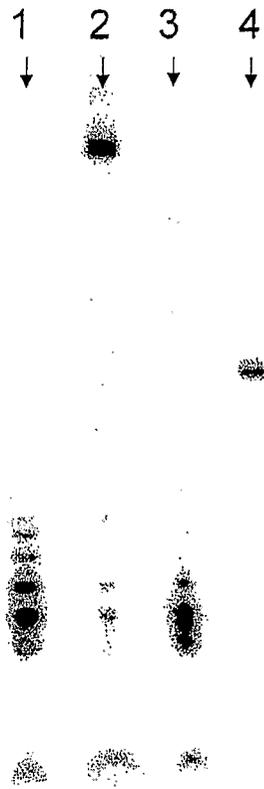


Fig. 12

Incorporation of LNA-A by 9° N DNA Polymerase

Primer	5'-TAATACGACTCACTATAGGCC-3'	37 ↓	21
Template	3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'		43

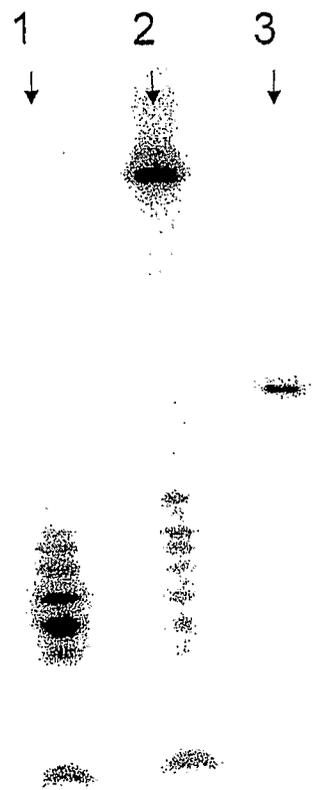


1. Positive control – using all four natural dNTPs
2. dATP was replaced with LNA-ATP, 43nt long
3. Negative control – dATP was absent in the reaction mix
4. Primer, 21nt long

Fig. 13

Incorporation of both LNA- T & A by 9° N DNA Polymerase

Primer	5'-TAATACGACTCACTATAGGCC-3'	21
Template	3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'	43



1. Positive control – using all four natural dNTPs
2. dATP and dTTP was replaced with LNA-ATP and LNA-TTP, 43nt long
3. Primer, 21nt long

Fig. 14

PCR using LNA-ATP

Forward Primer: 5'-TAATACGACTCACTATAGGCC-3' (21n, $T_m = 60\text{ }^\circ\text{C}$)

Reverse Primer: 3'-GACCCACACCTGGTCTGG-5' (18n, $T_m = 60\text{ }^\circ\text{C}$)

Template Sequence:

3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5' (43n)

Our System:

5'-TAATACGACTCACTATAGGCC-3'

3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'

3'-GACCCACACCTGGTCTGG-5'

15/51

PCR using LNA-ATP

Our System:

5'-TAATACGACTCACTATAGGCC-3'
3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'
3'-GACCCACACCTGGTCTGG-5'

PCR products:

5'-TAATACGACTCACTATAGGCCCGGCTGGGTGTGGACCAGACC-3'
3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'

5'-TAATACGACTCACTATAGGCCCGGCTGGGTGTGGACCAGACC-3'
3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'

5'-TAATACGACTCACTATAGGCCCGGCTGGGTGTGGACCAGACC-3'
3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'

5'-TAATACGACTCACTATAGGCCCGGCTGGGTGTGGACCAGACC-3'
3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'

Fig. 16

PCR using LNA-ATP by Phusion Polymerase

- PCR master mix:

Component	Volume/ 20 μ L reaction	Final Conc.
5 \times Phusion HF buffer	4.0 μ L	1 \times
MnCl ₂ , 50 mM	1.0 μ L	2.5 mM
Betaine enhancer, 2 M	1.0 μ L	0.1 M
dNTPs, 10 Mm	1.0 μ L	500 μ M
Primer A, 10 μ M	0.8 μ L	0.4 μ M
Primer B, 10 μ M	0.8 μ L	0.4 μ M
Template DNA, 10 μ M	0.1 μ L	0.05 μ M
H ₂ O	10.5 μ L	
Phusion HF DNA Polymerase	0.8 μ L	0.08 U/ μ L

Fig. 17

17/51

PCR using LNA-ATP by Phusion Polymerase

- PCR cycle parameters used:

Cycle step	Temp.	Time	Number of cycles
Initial denaturation	98 °C	30 sec	1
Denaturation Annealing Extension	98 °C 55 °C 72 °C	10 sec 30 sec 30 sec	25
Final extension	72 °C 4 °C	10 min Hold	1

Fig. 18

PCR using LNA-ATP by Phusion Polymerase

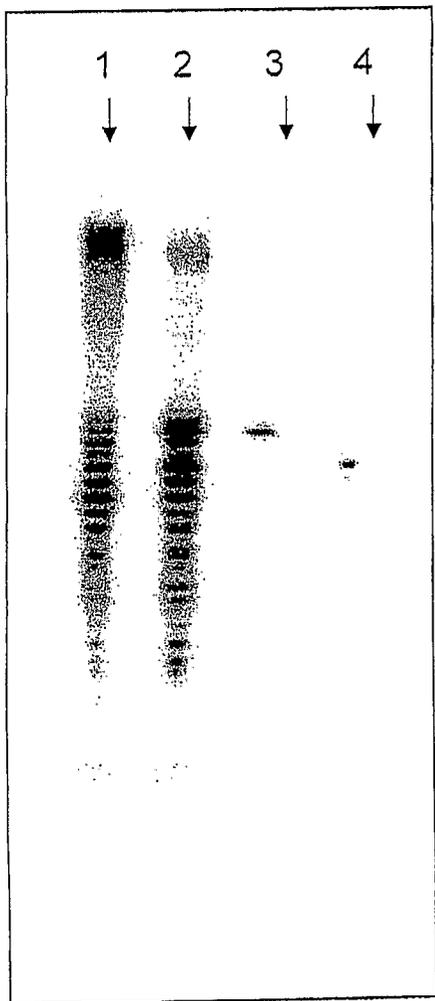


Fig. 19

1. Positive control – using all four natural dNTPs, 43nt long
2. dATP was replaced with LNA-ATP, 43nt long
3. Forward primer, 21nt
4. Reverse primer, 18nt

PCR using LNA-ATP by Phusion Polymerase

- PCR cycle parameters used:

Cycle step	Temp.	Time	Number of cycles
Initial denaturation	98 °C	5 min	1
Denaturation Annealing Extension	98 °C 50 °C 72 °C	4 min 5 min 20 min	20
Final extension	72 °C 4 °C	25 min Hold	1

Fig. 20

PCR using LNA-ATP by Phusion Polymerase

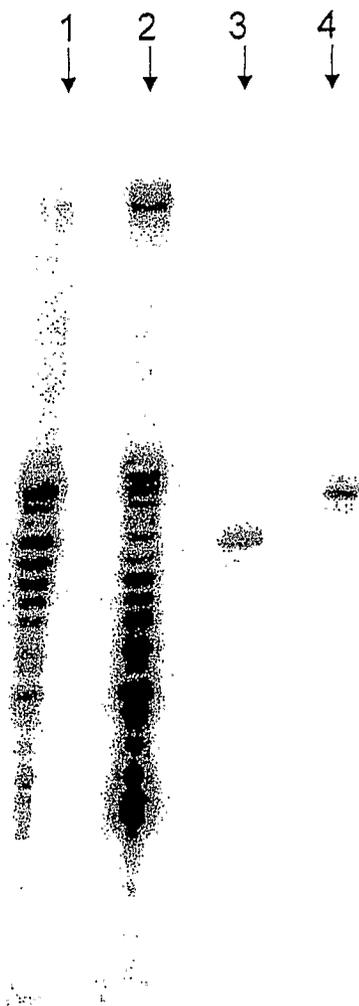


Fig. 21

1. Positive control – using all four natural dNTPs, 43nt long
2. dATP was replaced with LNA-ATP, 43nt long
3. Reverse primer, 18nt
4. Forward primer, 21nt

PCR using LNA-ATP, Comparative study of Phusion and 9N DNA polymerases

- PCR master mix:

Components	Volume/20 μ L reaction	Final Conc.
10 \times Thermopol buffer	2.0 μ L	1
MnCl ₂ , 50 mM	1.0 μ L	2.5 mM
Betaine enhancer, 2 M	1.0 μ L	0.1 M
dNTPs, 10 mM	1.5 μ L	750 μ M
Primer A, 50 pmol	0.35 μ L	0.87 μ M
Primer B, 50 pmol	0.35 μ L	0.87 μ M
Template DNA, 0.5 μ M	0.25 μ L	0.006 μ M
H ₂ O	12.70 μ L	
9 ^o N DNA polymerase	1.0 μ L	0.08 / μ L

Fig. 22

PCR using LNA-ATP, Comparative study of Phusion and 9N DNA polymerases

PCR System:

5'-TAATACGACTCACTATAGGCC-3'

3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'

3'-GACCCACACCTGGTCTGG-5'

dNTP mix: dCTP, dGTP, LNA-ATP, dTTP

Initial denaturation	98 °C	30 Sec	1 cycle
Denaturation	98 °C	10 Sec	19 cycles
Annealing	55 °C	30 Sec	
Extension	72 °C	35 Sec	
Final extension	72 °C	10 min	
Hold	4 °C	—	

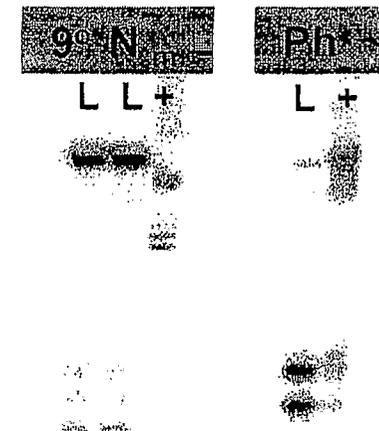


Fig. 23

Total cycle time: 60 minutes

PCR using LNA-ATP using 9N DNA polymerase

PCR System:

5'-TAATACGACTCACTATAGGCC-3'

3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'

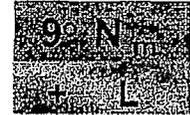
3'-GACCCACACCTGGTCTGG-5'

dNTP mix: dCTP, dGTP, LNA-ATP, dTTP

Initial denaturation	98 °C	30 Sec	1 cycle
Denaturation	98 °C	10 Sec	19 cycles
Annealing	55 °C	30 Sec	
Extension	72 °C	35 Sec	
Final extension	72 °C	10 min	
Hold	4 °C		

Total cycle time: 60 minutes

Fig. 24



PCR results - Using LNAT and 9N Polymerases



1. Positive control – using all natural dNTPs, 43n
2. dTTP was replaced with LNA-TTP, 43n
3. dATP was replaced with LNA-ATP, 43n
4. 43n long DNA control
5. Forward primer, 21nt
6. Reverse primer, 18nt

Fig. 25

PCR experiment by using both LNA-A & T

PCR System:

5'-TAATACGACTCACTATAGGCC-3'

3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'

3'-GACCCACACCTGGTCTGG-5'

dNTP mix: dCTP, dGTP, LNA-ATP, LNA-TTP

Initial denaturation	98 °C	30 Sec	1 cycle
Denaturation	98 °C	10 Sec	19 cycles
Annealing	55 °C	30 Sec	
Extension	72 °C	35 Sec	
Final extension	72 °C	10 min	
Hold	4 °C	-	

Total cycle time: 60 minutes

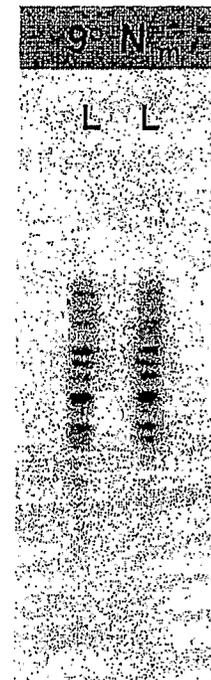


Fig. 26

PCR experiment without Annealing step using LNA-ATP and 9N polymerase

PCR System:

5'-TAATACGACTCACTATAGGCC-3'

3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'

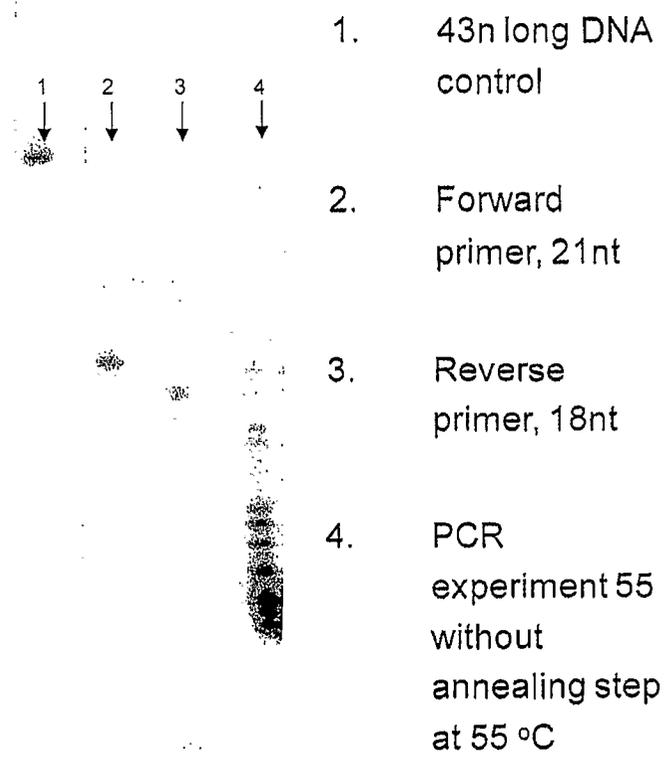
3'-GACCCACACCTGGTCTGG-5'

dNTP mix: dCTP, dGTP, LNA-ATP, dTTP

Fig. 27

Initial denaturation	98 °C	30 Sec	1 cycle
Denaturation	98 °C	10 Sec	
Extension	72 °C	35 Sec	19 cycles
Final extension	72 °C	10 min	
Hold	4 °C	-	

Total cycle time: 38 minutes



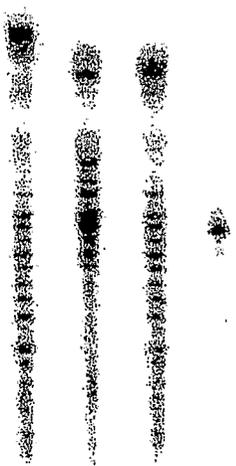
Incorporation of N-methyl amino LNA-TTP

Incorporation of N-methyl amino LNA-TTP		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCCTTCTGGGGATAGACGG-5'	44

37
↓

Phusion HF polymerase

1 2 3 4



1. Positive control – using all four natural dNTPs, 44n
2. dTTP was replaced with N-Me-LNA-TTP, 36n
3. Negative control - dCTP, dGTP, dATP in the mix, 36n
4. Primer, 19nt long

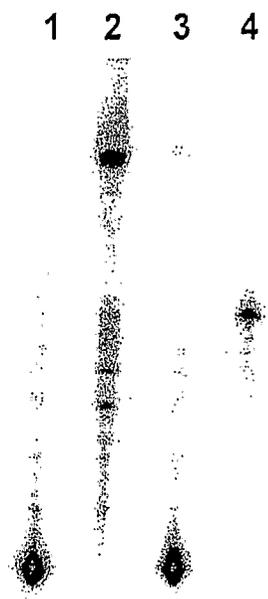
Fig. 28

Incorporation of N-methyl amino LNA-TTP

Incorporation of N-methyl amino LNA-TTP		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGGATAGACGG-5'	44

37
↓

9 N polymerase



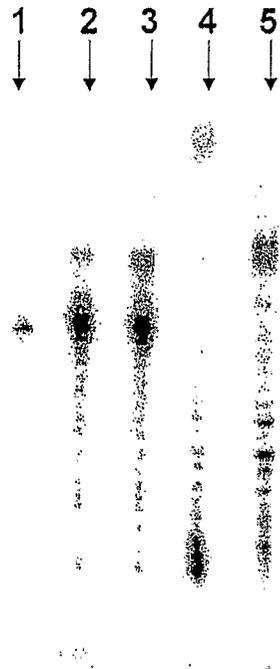
1. Positive control – using all four natural dNTPs, 44n
2. dTTP was replaced with N-Me-LNA-TTP
3. Negative control - dCTP, dGTP, dATP in the mix, 36n
4. Primer, 19nt long

Fig. 29

Incorporation of N-methyl amino LNA-TTP

Incorporation of N-methyl amino LNA-TTP			Length
Primer	5'-TAATACGACTCACTATAGG-3'	27 ↓	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCG <u>ACCCAC</u> CCTGGTCTGG-5'		43

Fig. 30



Phusion HF polymerase

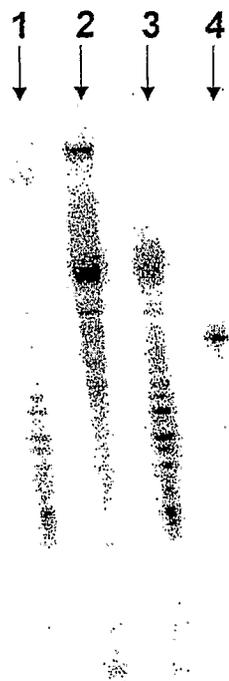
1. Primer, 19nt long
2. dTTP was replaced with N-Me-LNA-TTP, 26n. Old polymerase
3. dTTP was replaced with N-Me-LNA-TTP, 26n. New polymerase
4. Positive control – using all four natural dNTPs, 43n.
5. Negative control - dCTP, dGTP, dATP in the mix, 26n.

30/51

Incorporation of N-acetyl amino LNA-TTP

Incorporation of N-acetyl amino LNA-TTP		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCG <u>A</u> CC <u>C</u> A <u>C</u> CTGGTCTGG-5'	43

Phusion HF polymerase



1. Positive control – using all four natural dNTPs, 43n.
2. dTTP was replaced with N-acetyl-LNA-TTP
3. Negative control - dCTP, dGTP, dATP in the mix, 26n
4. Primer, 19nt long

Fig. 31

Incorporation of N-acetyl amino LNA-TTP

Incorporation of N-acetyl amino LNA-TTP

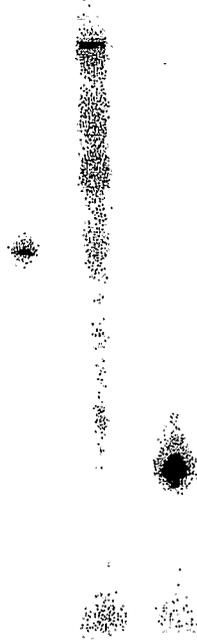
Primer 5'-TAATACGACTCACTATAGG-3'

27

Template 3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'

9N polymerase

1
2
3

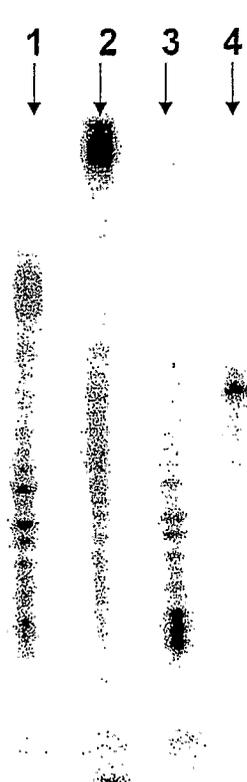


1. Primer, 19nt long
2. dTTP was replaced with N-acetyl amino-LNA-TTP
3. Positive control – using all four natural dNTPs, 43n

Fig. 32

Incorporation of amino LNA-TTP

<u>Incorporation of amino LNA-TTP</u>			Length
Primer	5'-TAATACGACTCACTATAGG-3'	27 ↓	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCG <u>A</u> CC <u>C</u> A <u>C</u> CCTGGTCTGG-5'		43



Phusion HF polymerase

1. Negative control - dCTP, dGTP, dATP in the mix, 26n
2. dTTP was replaced with amino-LNA-TTP
3. Positive control – using all four natural dNTPs, 43n
4. Primer, 19nt long

Fig. 33

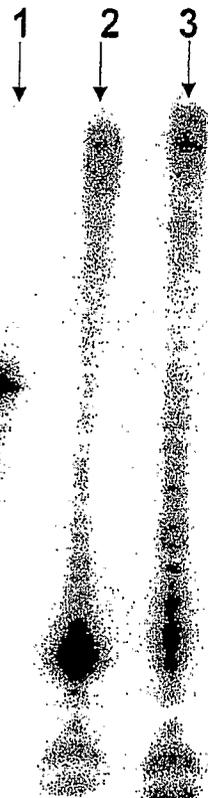
Incorporation of amino LNA-TTP

Incorporation of amino LNA-TTP		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCG <u>A</u> CC <u>C</u> AC <u>A</u> CCTGGTCTGG-5'	43

27



9N polymerase



1. Primer, 19nt long
2. Positive control – using all four natural dNTPs, 43n
3. dTTP was replaced with amino LNA-TTP

Fig. 34

34/51

Incorporation of amino LNA-TTP

Incorporation of amino LNA-TTP		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGGATAGACGG-5'	44

37
↓

Phusion HF polymerase



Fig. 35

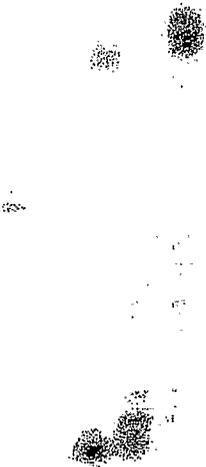
1. Primer, 19nt long
2. Negative control - dCTP, dGTP, dATP in the mix, 36n
3. dTTP was replaced with LNA-TTP, 44nt long
4. Positive control – using all four natural dNTPs, 44nt long

Incorporation of amino LNA-TTP

Incorporation of amino LNA-TTP		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGG <u>A</u> <u>T</u> <u>A</u> <u>G</u> <u>A</u> CGG-5'	44

9N polymerase

1 2 3 4
↓ ↓ ↓ ↓



1. Primer, 19nt long
2. Negative control - dCTP, dGTP, dATP in the mix, 36n
3. Positive control – using all four natural dNTPs
4. dTTP was replaced with LNA-TTP, 44nt long

Fig. 36

Incorporation of LNA-T

Primer	5'-TAATACGACTCACTATAGG-3'	37	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGGATAGACGG-5'	↓	44

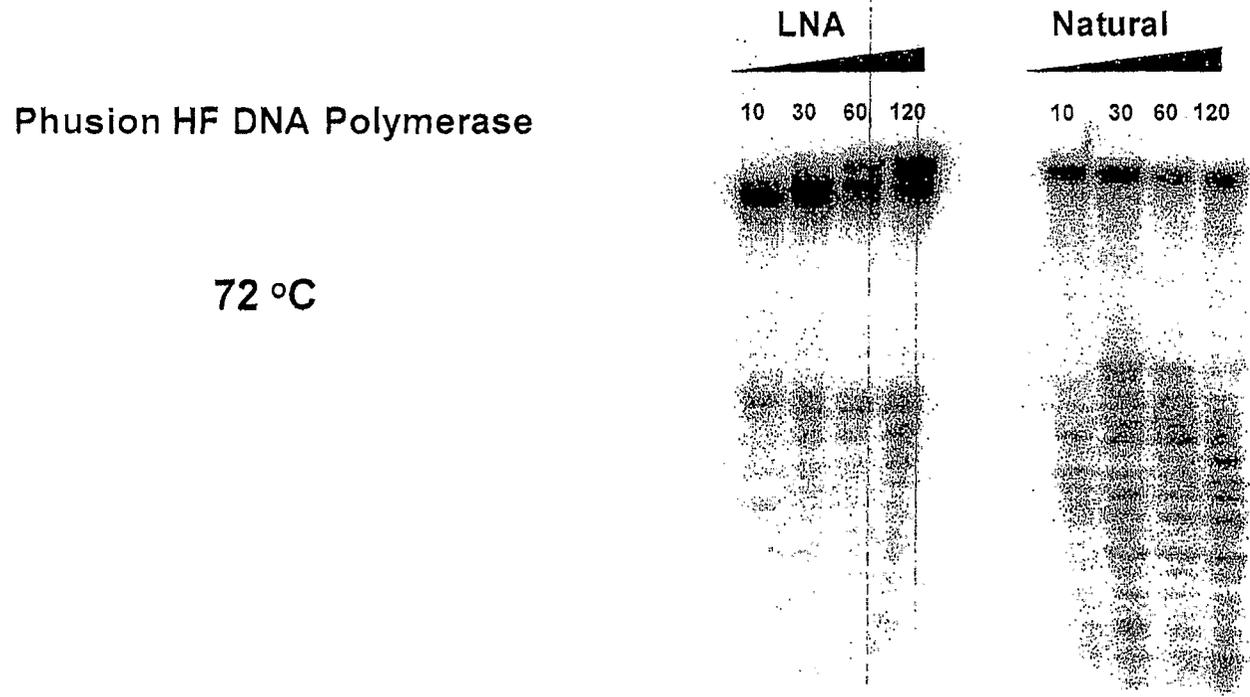
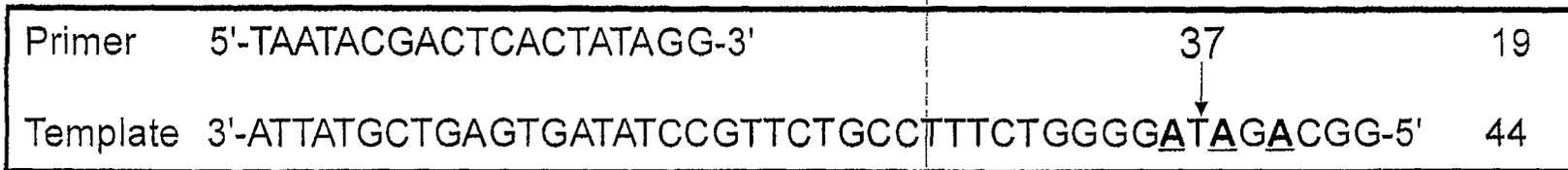


Fig. 37

Incorporation of LNA-T



9N polymerase

72 °C

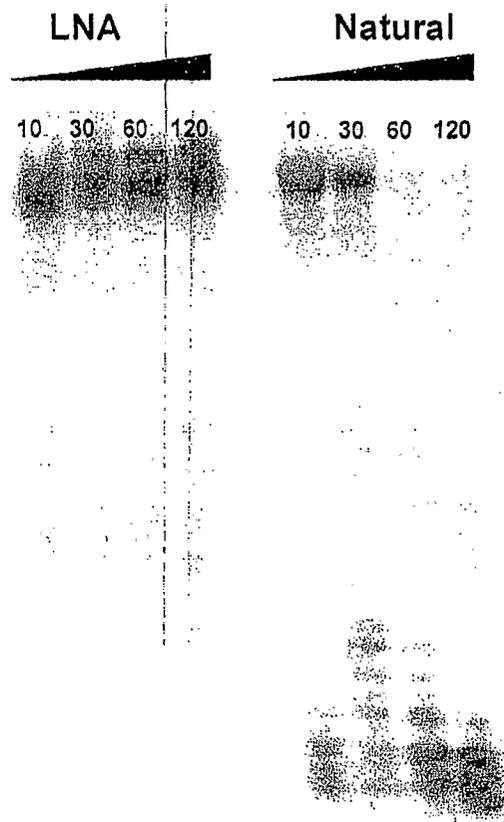


Fig. 38

Incorporation of N-acetyl amino LNA-TTP

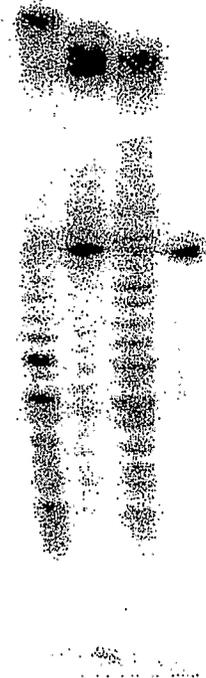
Incorporation of N-acetyl amino LNA-TTP		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGG <u>A</u> <u>T</u> <u>A</u> <u>G</u> <u>A</u> CGG-5'	44

37



Phusion HF polymerase

1 2 3 4
↓ ↓ ↓ ↓



1. Positive control – using all four natural dNTPs, 44nt long
2. dTTP was replaced with LNA-TTP
3. Negative control - dCTP, dGTP, dATP in the mix, 36n
4. Primer, 19nt long

Fig. 39

Incorporation of N-acetyl amino LNA-TTP

Incorporation of N-acetyl amino LNA-TTP			Length
Primer	5'-TAATACGACTCACTATAGG-3'	37 ↓	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCC	TTTCTGGGG <u>A</u> T <u>A</u> G <u>A</u> CGG-5'	44

1
↓
2
↓
3
↓

9N polymerase



1. Positive control – using all four natural dNTPs, 44nt long
2. dTTP was replaced with LNA-TTP
3. Primer, 19nt long

Fig. 40

Fidelity of amino LNA-TTP incorporation

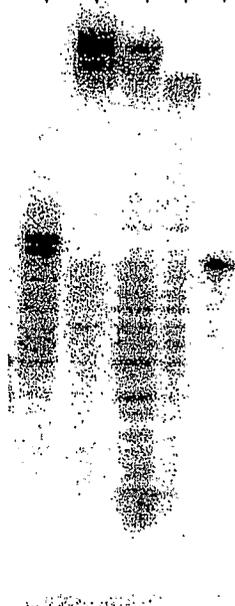
Fidelity using amino LNA-TTP		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGGATAGACGG-5'	44

20



Phusion HF polymerase

1 2 3 4 5
↓ ↓ ↓ ↓ ↓



1. Fidelity set – dCTP, dGTP and LNA-TTP only in the mix, 20-21nt
2. dTTP was replaced with LNA-TTP, 44nt long
3. Positive control – using all four natural dNTPs, 44nt long
4. Negative control - dCTP, dGTP, dATP in the mix, 36n
5. Primer, 19nt long

Fig. 41

Incorporation of amino LNA-T on LNA modified template

Incorporation of amino LNA-T		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCGACCC <u>ACACCTGGICTGG</u> -5'	43

27
↓

Fig. 42

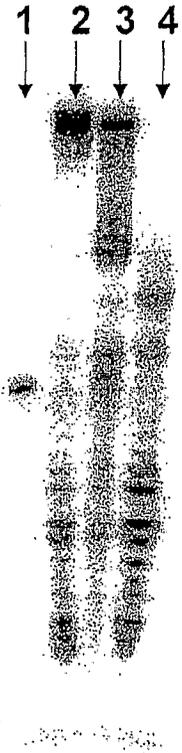


Phusion HF polymerase

1. Negative control – dTTP and dATP was absent in the reaction mix, 26n.
2. dTTP was replaced with amino LNA-TTP, 43n
3. Positive control – using all four natural dNTPs, 43n
4. Primer, 19n

Incorporation of amino LNA-T and LNA-A nucleotides together		Length
Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCGACCC <u>A</u> C <u>A</u> CTGGTCTGG-5'	43

Phusion HF polymerase



1. Primer, 19n
2. Positive control – using all four natural dNTPs, 43n
3. dTTP and dATP was replaced with amino LNA-TTP and LNA-ATP, 43n
4. Negative control – dTTP and dATP was absent in the reaction mix, 26n

Fig. 43

43/51

44/51

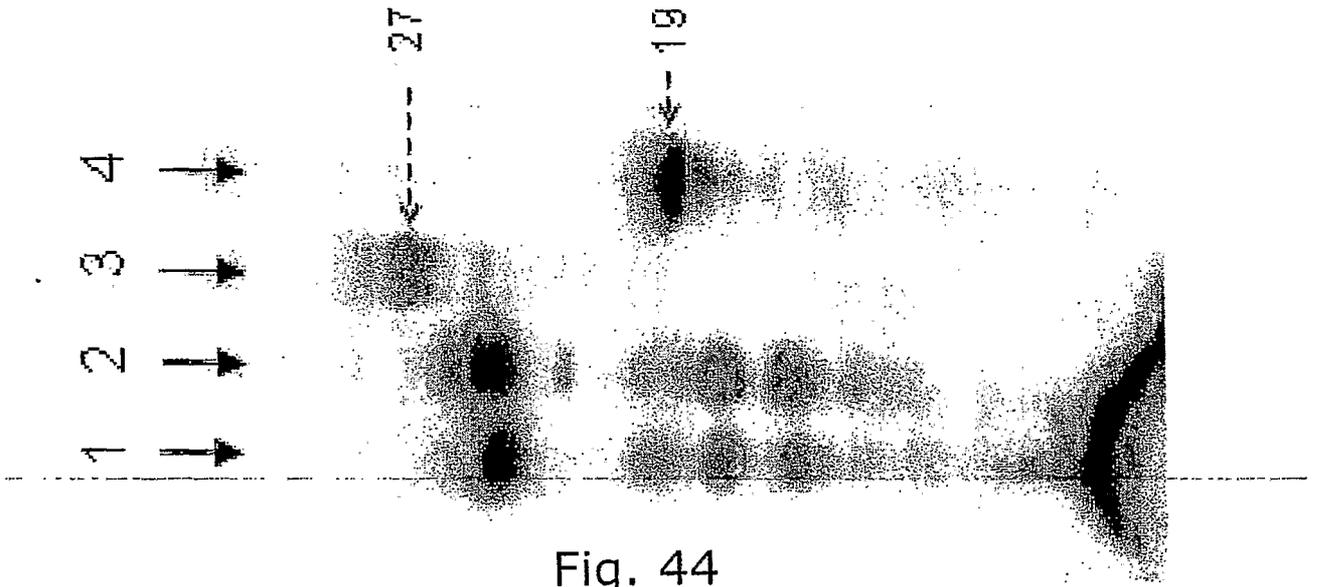
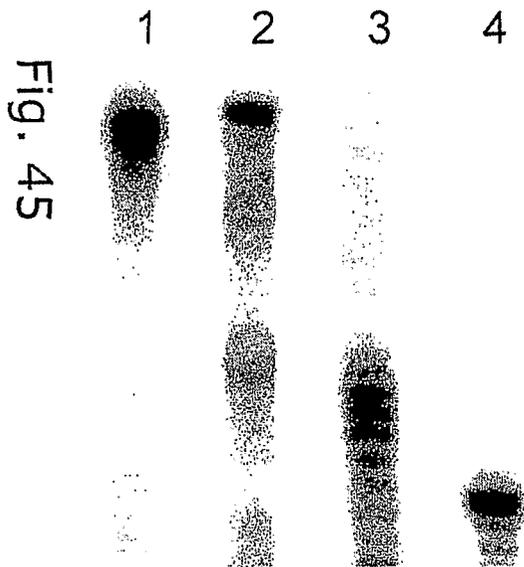


Fig. 44

Incorporation of LNA-A, LNA-T & LNA-mC

KOD Dash DNA polymerase

Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCCC <u>TCTC</u> <u>ACGC</u> <u>ACGT</u> <u>GTAGTCC</u> -5'	40



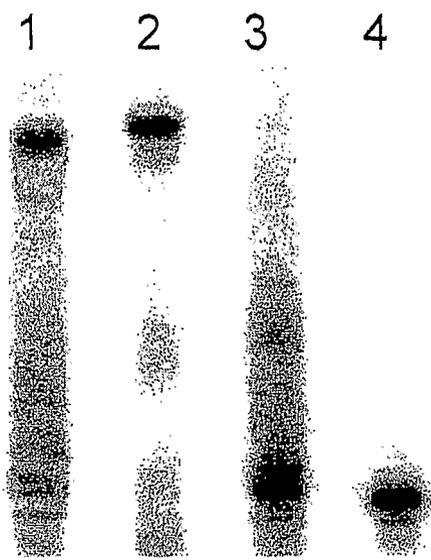
1. Positive control – using all four natural dNTPs
2. dCTP, dATP and dTTP was replaced with corresponding LNA modifications
3. Negative control – dCTP, dATP and dTTP was absent in the reaction mix

45/51

Successive Incorporation of LNA-mC

KOD Dash DNA polymerase

Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCTATTTTCACGGGGGGGGCACTTATCC	-5' 44



1. Positive control – using all four natural dNTPs
2. dCTP was replaced with corresponding LNA-modification
3. Negative control – dCTP absent in the mix
4. Primer, 19n

SUBSTITUTE SHEET (RULE 26)

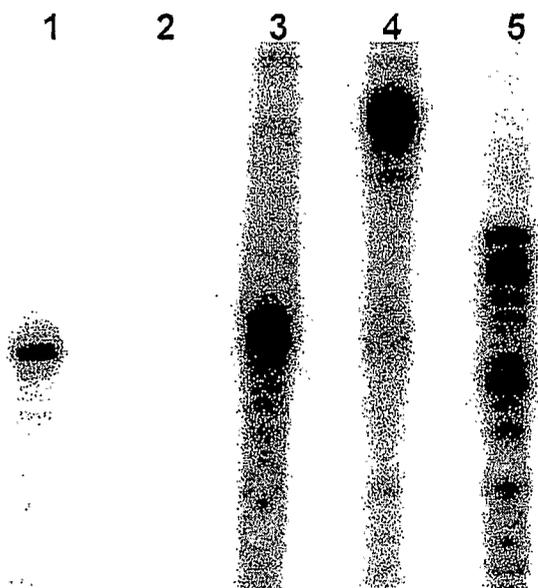
Fig. 46

46/51

Incorporation of LNA- T, LNA-A and LNA-mC

KOD DNA Polymerase

Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCTATGTATGTGTATGT-5'	34



1. Primer, 19n
2. Control 34n DNA
3. Negative control – dTTP, dATP and dCTP were absent in the reaction mix
4. Positive control – using all natural dNTPs
5. dTTP, dATP and dCTP were replaced with corresponding LNA-modifications

Fig. 47

PCR USING KOD DNA POLYMERASE

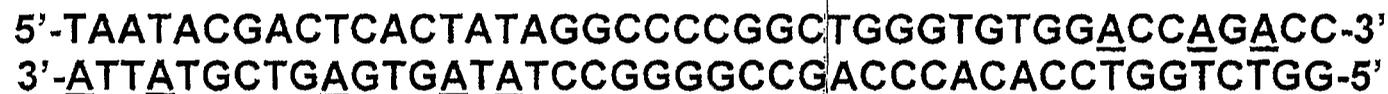
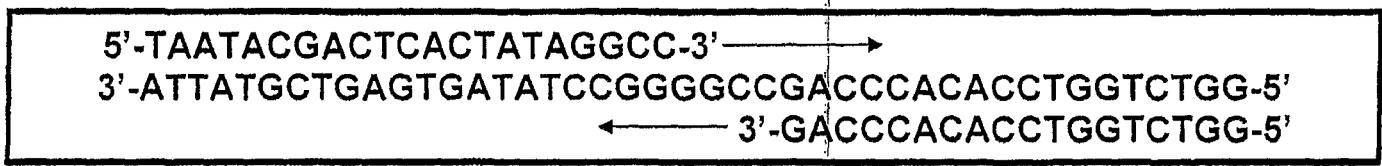


Fig. 48

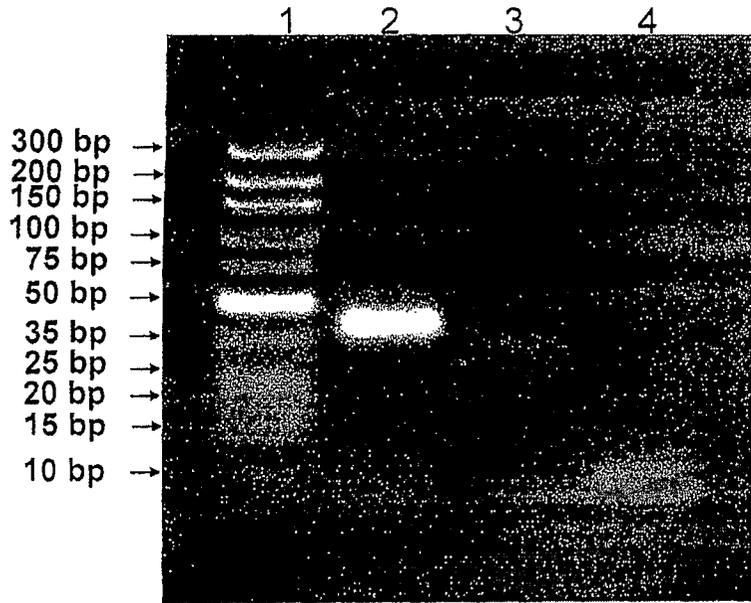
Components	Volume
MgSO 25 mM	
BSA, 2.5 mg/mL	
dNTPs, 2.5 mM mix	
H ₂ O, dd	
Primer A, 50 pmol	
Primer B, 50 pmol	
Template DNA, 4 pmol	
KOD DNA polymerase	

Initial denaturation	95 °C	15 Sec	1 cycle
Denaturation	95 °C	1 Sec	30 cycles
Annealing	60 °C	5 Sec	
Extension	72 °C	5 Sec	
Hold	4 °C	-	-

PCR USING KOD DNA POLYMERASE

PCR product:

5'-TAATACGACTCACTATAGGCCCCGGCTGGGTGTGGACCAGACC-3'
 3'-ATTATGCTGAGTGATATCCGGGGCCGACCCACACCTGGTCTGG-5'



1. DNA ladder (10-300 bp, 5 µg)
2. PCR using LNA-ATP
3. PCR using dNTPs
4. LNA-PCR without polymerase

Fig. 49

PCR USING KOD DNA POLYMERASE

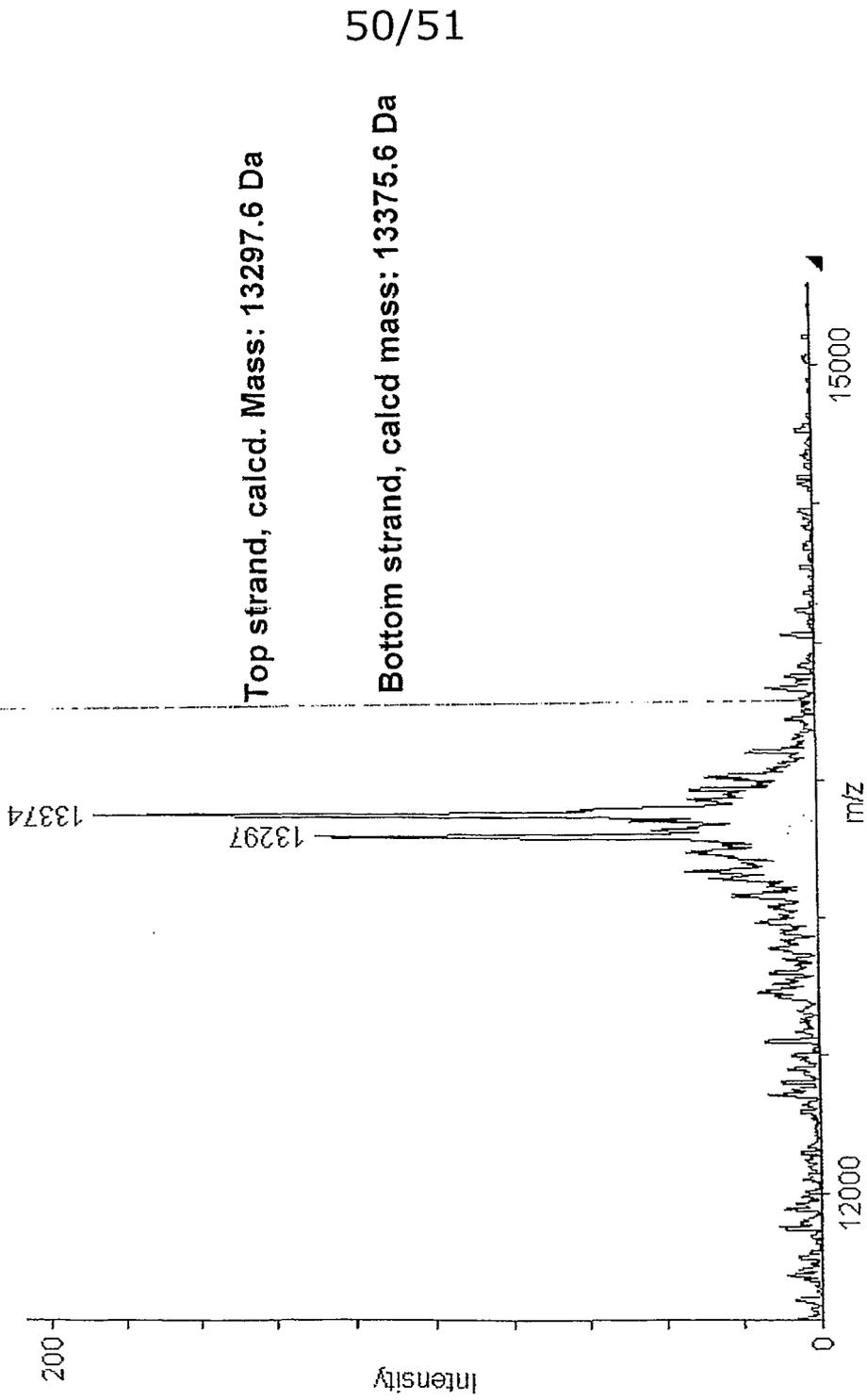


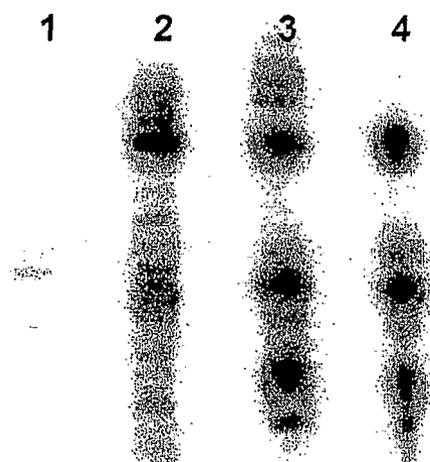
Fig. 50

Transcription - Incorporation of LNA-U

T7 RNA polymerase

Primer	5'-TAATACGACTCACTATAGG-3'	19
Template	3'-ATTATGCTGAGTGATATCCGGGGCCG <u>A</u> CC <u>C</u> <u>A</u> CCTGGTCTGG-5'	43

5'-GGCCCCGGCUGGGUGUGGACCAGACC-3' (26n transcript)



- 19n primer
- 26n RNA marker
- Using natural rNTPs
- UTP is replaced with **LNA-UTP**

Fig. 51