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(54) Title: QUANTIFICATION OF ANTI SENSE OLIGONUCLEOTIDES (ASOs)

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QUANTIFICATION OF ANTI SENSE OLIGONUCLEOTIDES (ASOs)

Description

The present invention relates to methods and kits for determining oligonucleotide analytes in a sample, more particularly for quantitatively determining an antisense oligonucleotide analyte in a biological sample.

Antisense oligonucleotides are synthetic single-stranded nucleic acid molecules that are capable to bind to complementary target RNA molecules by hybridization. Thereby, antisense oligonucleotides interfere with gene expression.

Antisense oligonucleotides have been developed as drugs for diseases such as cancers including lung cancer, colorectal carcinoma, pancreatic carcinoma, malignant glioma and malignant melanoma, diabetes, amyotrophic lateral sclerosis (ALS), Duchenne muscular dystrophy, spinal muscular atrophy, asthma, arthritis and other diseases. Meanwhile, several antisense drugs have been approved by the US Food and Drug Administration.

Numerous methods for determining the amount of analytes including single-stranded oligonucleotides are known in the art. A quantitative determination of oligonucleotide analytes in biological samples, however, is still challenging.

In view of this, there is a high need for providing an efficient method for determining the presence and amount of antisense oligonucleotides in samples, particularly in vivo in preclinical and clinical samples.

US 5,770,365 discloses a method for promoting the hybridization of a target single-stranded nucleic acid and a nucleic acid-capturing moiety which is formed as an immobilized hairpin structure. An oligonucleotide analyte hybridizes to the immobilized hairpin resulting in the formation of an immobilized nicked double strand comprising an intramolecular and an intermolecular portion. Determination of the analyte is possible by several methods.

US 6,355,438 B1 discloses a method for detecting or quantitating an oligonucleotide analyte, wherein said oligonucleotide comprises at least one modification. The method comprises forming a hybrid of the modified oligonucleotide with a probe, wherein the probe comprises a region at one end thereof which does not hybridize to the modified oligonucleotide and subsequently incorporating a detectable label into the modified oligonucleotide opposite to the non-hybridizing region of the probe by means of a ligation reaction between the oligonucleotide analyte with a labelled detection probe and detecting the label.

US 7,306,904 B2 discloses an assay for detecting and/or quantitating one or several analytes in solution by a proximity probe, comprising an analyte binding moiety coupled to an oligonucleotide with a free 5'-end and a further proximity probe comprising a further analyte binding moiety coupled to an oligonucleotide with a free 3'-end. The oligonucleotides from different proximity probes are capable of interaction with each other, e.g. by ligation, when bound via the binding moieties to the analyte. The ligation product may be detected by nucleic acid amplification.

The present invention provides novel methods and kits which allow quantitative determination of oligonucleotide analytes, e.g. antisense oligonucleotides, through a ligation/amplification reaction combining the sensitivity of an amplification with the specificity of a ligase reaction. The oligonucleotide analyte serves as a junction or splint bridging two

complementary nucleic acid sequence portions present in one or more ligation partner oligonucleotides by sequence-dependent Watson-Crick base pairing. Hybridization of the oligonucleotide analyte with the two complementary nucleic acid sequence portions of the ligation partner(s) generates a contiguous sequence of the two complementary sequence portions interrupted by a nick. The respective ends of the sequence portions have ligation-accessible 5'- and 3'-ends allowing that the nick is converted to a covalent bond, e.g. a phosphodiester bond by an enzymatic ligation. The product of the ligation reaction, i.e. ligation product, comprises the two complementary sequence portions of the ligation partner(s) covalently linked to each other. The amount of ligation product correlates with the amount of oligonucleotide analyte present in the sample. Thus, the ligation product may serve as an amplification template allowing a quantitative determination of the analyte by nucleic acid amplification, e.g. by real time quantitative PCR (qPCR).

In one aspect, the present invention relates to a method for determining an oligonucleotide analyte in a sample, comprising

(i) adding to the sample at least one ligation partner of the oligonucleotide analyte,

wherein the at least one ligation partner is an oligonucleotide capable of hybridizing with the oligonucleotide analyte,

(ii) forming a hybridization product between the oligonucleotide analyte and the at least one ligation partner, wherein the oligonucleotide analyte bridges together two ends of the at least one ligation partner wherein a contiguous sequence of nucleotides interrupted by a nick between a ligation-accessible 3'-end and a ligation-accessible 5'-end of the at least one ligation partner is generated,

(iii) closing the nick by a ligation reaction, wherein a covalent bond, particularly a phosphodiester bond, between the accessible 3'-end and the accessible 5'-end of the at least one ligation partner is formed,

(iv) subjecting the ligation product to a nucleic acid amplification reaction, and

(v) qualitatively or quantitatively determining the product of amplification reaction.

In a further aspect, the present invention relates to a kit for determining an oligonucleotide analyte in a sample comprising:

(a) optionally an oligonucleotide analyte,

(b) at least one ligation partner of the oligonucleotide analyte,

wherein the oligonucleotide analyte is capable of hybridizing with the at least one ligation partner by bridging together two ends of the at least one ligation partner wherein a contiguous sequence of nucleotides interrupted by a nick between a ligation-accessible 3'-end and a ligation-accessible 5'-end of the at least one ligation partner is generated,

(c) optionally a ligase,

(d) optionally primers and/or stacking partners, and

(e) optionally an instruction manual.

In a further aspect, the present invention relates to a method for determining an oligonucleotide analyte in a sample comprising

(i) adding to the sample at least one ligation partner of the oligonucleotide analyte,

wherein the at least one ligation partner is an oligonucleotide capable of hybridizing with the oligonucleotide analyte,

(ii) forming a hybridization product between the oligonucleotide analyte and the at least one ligation partner, wherein the oligonucleotide analyte bridges together two ends of the at least one ligation partner wherein a contiguous sequence of nucleotides interrupted by a nick between a ligation-accessible 3'-end and a ligation-accessible 5'-end of the at least one ligation partner is generated,

(iii) closing the nick by a ligation reaction, wherein a covalent bond, particularly a phosphodiester bond, between the accessible 3'-end and the accessible 5'-end of the at least one ligation partner is formed,

(iv) subjecting the ligation product to a nucleic acid amplification reaction, and

(v) qualitatively or quantitatively determining the product of amplification reaction,

wherein the oligonucleotide analyte is an antisense oligonucleotide, e.g. an antisense oligonucleotide comprising at least one modified nucleoside building block, particularly a locked nucleoside building block, and/or at least one modified internucleosidic linkage, particularly a phosphorothioate linkage.

In a further aspect, the invention relates to a method for determining an oligonucleotide analyte in a sample, comprising

(i) adding to the sample at least one ligation partner of the oligonucleotide analyte,

wherein the at least one ligation partner is an oligonucleotide capable of hybridizing with the oligonucleotide analyte,

(ii) forming a hybridization product between the oligonucleotide analyte and the at least one ligation partner, wherein the oligonucleotide analyte bridges together two ends of the at least one ligation partner wherein a contiguous sequence of nucleotides interrupted by a nick between a ligation-accessible 3'-end and a ligation-accessible 5'-end of the at least one ligation partner is generated,

(iii) closing the nick by a ligation reaction, wherein a covalent bond, particularly a phosphodiester bond, between the accessible 3'-end and the accessible 5'-end of the at least one ligation partner is formed,

(iv) subjecting the ligation product to a nucleic acid amplification reaction, and

(v) qualitatively or quantitatively determining the product of amplification reaction,

wherein the method comprises adding to the sample a first ligation partner and a second ligation partner, wherein the first ligation partner comprises a 3'-terminal sequence portion with a ligation-accessible 3'-end which hybridizes with a 3'-terminal sequence portion of the oligonucleotide analyte and the second ligation partner comprises a 5'-terminal sequence portion with a ligation-accessible 5'-end which hybridizes with a 5'-terminal sequence portion of the oligonucleotide analyte thereby forming a linear contiguous sequence interrupted by a nick, and

wherein the first ligation partner having a ligation-accessible 3'-end comprises at least one modified nucleoside building block, particularly at least one locked nucleoside building block, and the second ligation partner having a ligation-accessible 5'-end does not comprise a modified nucleoside building block, particularly a locked nucleoside building block.

In a further aspect, the invention relates to a method for determining an oligonucleotide analyte in a sample, comprising

(i) adding to the sample at least one ligation partner of the oligonucleotide analyte,

wherein the at least one ligation partner is an oligonucleotide capable of hybridizing with the oligonucleotide analyte,

(ii) forming a hybridization product between the oligonucleotide analyte and the at least one ligation partner, wherein the oligonucleotide analyte bridges together two ends of the at least one ligation partner wherein a contiguous sequence of nucleotides interrupted by a nick between a ligation-accessible 3'-end and a

ligation-accessible 5'-end of the at least one ligation partner is generated,

(iii) closing the nick by a ligation reaction, wherein a covalent bond, particularly a phosphodiester bond, between the accessible 3'-end and the accessible 5'-end of the at least one ligation partner is formed,

(iv) subjecting the ligation product to a nucleic acid amplification reaction, and

(v) qualitatively or quantitatively determining the product of amplification reaction,

wherein at least one stacking partner of the oligonucleotide analyte is added to the sample before step (iii), wherein the stacking partner is an oligonucleotide which hybridizes with the at least one ligation partner adjacent to the 3'-end and/or the 5'-end of the oligonucleotide analyte.

The method and kit of the invention are suitable for determining an oligonucleotide analyte in a sample. The term "determining" comprises qualitatively determining the presence of the oligonucleotide analyte and/or quantitatively determining the amount of the oligonucleotide analyte in the sample. Preferably, the present invention comprises a quantitative determination.

The term "oligonucleotide analyte" relates to a single-stranded oligonucleotide which may have a length of about 8 to about 50 nucleotides, preferably about 10 to about 25 nucleotides. The oligonucleotide analyte may be a DNA and/or RNA oligonucleotide optionally comprising at least one modified nucleosidic building block and/or at least one modified internucleosidic linkage between two nucleoside building blocks.

In particular embodiments, the oligonucleotide analyte is an antisense oligonucleotide. The term "antisense oligonucleotide" relates in particular to

oligodeoxyribonucleotides which may comprise at least one modified nucleoside building block and/or at least one modified internucleosidic linkage. The presence of modified nucleoside building blocks and/or modified internucleosidic linkages increases the stability of antisense oligonucleotides under physiological conditions. Thus, pharmaceutical antisense oligonucleotides usually comprise such modifications.

Examples of modified nucleoside building blocks are base-modified building blocks comprising a non-naturally occurring nucleobase, e.g. 5-methyl cytosine, and sugar-modified building blocks comprising a non-naturally occurring sugar moiety, e.g. a 2'-modified sugar moiety and/or a locked sugar moiety. Examples of 2'-modified nucleoside building blocks are 2'-methoxy, 2'-F and/or 2'-O-ethoxymethyl building blocks. Locked nucleoside building blocks contain a bridge between two atoms of the pentose sugar, e.g. the deoxyribose sugar. For example, a bridge is formed between the 2'-C atom and the 4'-C atom of the sugar, e.g. a 2-atom or 3-atom bridge, preferably comprising at least one heteroatom such as O, N or S. Preferred are bridges having the structure 2'-O-CH_2-4' or 2'-O-CH_2-CH_2-4', wherein at least one H atom may be substituted, e.g. by C_1-C_3 alkyl, C_1-C_3 alkoxy or C_1-C_3 alkyl, or C_1-C_3 alkoxy groups, e.g. a 2'-O-CH(CH_3)-4' bridge. Especially preferred is a bridge having the structure 2'-O-CH_2-4'.

The internucleosidic linkages in the oligonucleotide analyte may be phosphodiester bonds and/or modified internucleosidic linkages such as phoshorothioate linkages.

In many cases, antisense oligonucleotides comprise a plurality of modified nucleoside building blocks, e.g. locked nucleoside building blocks and/or 2'-modified building blocks and a plurality of modified internucleosidic linkages.

Modified nucleoside building blocks and/or modified nucleosidic linkages may be present at any position of the antisense oligonucleotide. The respective

molecules are designated as gapmer, blockmer, headmer, tailmer, mixmer or totalmer. The present invention is applicable to all of these molecules since it does not require the presence of ligation-accessible 5'- and/or 3'-ends in the oligonucleotide analyte.

The method of the invention is used for determining an oligonucleotide analyte in a sample. The sample may be a biological sample, e.g. a cell culture, tissue and/or body fluid sample such as a tissue biopsy, blood, serum, plasma, urine sample, etc. The sample may be pretreated, e.g. by lysis of cellular components according to standard methods. The determination of the oligonucleotide analyte may be carried out directly in the lysed sample or in a fraction thereof.

In a biological sample, the oligonucleotide analyte is usually present together with endogenous nucleic acid molecules including genomic DNA and/or RNA molecules which may comprise the nucleotide sequence of the oligonucleotide analyte and/or its complement thereof. The method of the invention allows determination of the oligonucleotide analyte in the presence of endogenous nucleic acid molecules. Thus, the method allows determination of the oligonucleotide in a sample without previous separation of endogenous nucleic acid molecules present in the sample, particularly without previous separation of endogenous RNA molecules present in the sample. Thus, in one embodiment, the method comprises determination of the oligonucleotide in an RNA-containing sample fraction obtained from a biological sample.

Step (i) of the method of the invention comprises adding at least one ligation partner of the oligonucleotide analyte to the sample. The term "ligation partner" refers to an oligonucleotide comprising at least one sequence portion complementary to the nucleotide sequence of the analyte. Further, the ligation partner comprises a ligation-accessible 5'- and/or 3'-end, i.e. an end which is accessible to ligation wherein a covalent bond is formed.

In one embodiment, the invention involves the use of a first ligation partner and a second ligation partner which are both oligonucleotides capable of hybridzing with the oligonucleotide analyte. The first ligation partner comprises a 3'-terminal sequence portion with a ligation-accessible 3'-end which hybridizes with a 3'-terminal sequence portion of the oligonucleotide analyte. The second ligation partner comprises a 5'-terminal sequence portion with a ligation-accessible 5'-end which hybridizes with a 5'-terminal sequence portion of the oligonucleotide analyte. In case the oligonucleotide analyte is present in the sample, a double-stranded hybridization product comprising the analyte and the first and second ligation partners is formed in step (ii). A first strand of the hybridization product comprises the oligonucleotide analyte and the second strand of the hybridization product comprises the first and the second ligation partner. Since the first and second ligation partners comprise sequence portions which do not hybridize with the oligonucleotide analyte, the second strand extends on both sides beyond the ends of the first strand. The nucleotide sequences of the first and second ligation partners are selected such that they, when hybridized to the oligonucleotide analyte, form a linear contiguous sequence of nucleotides with a nick between the ligation-accessible 3'-end of the first ligation partner and the ligation-accessible 5'-end of the second ligation partner.

A further embodiment of the invention involves the use of only one ligation partner. In this case, the ligation partner comprises a 3'-terminal sequence portion with a ligation-accessible 3'-end which hybridizes with a 3'-terminal sequence portion of the oligonucleotide analyte and a 5'-terminal sequence with a ligation-accessible 5'-end which hybridizes with a 5'-terminal sequence portion of the oligonucleotide analyte thereby forming a circular contiguous sequence interrupted by a nick. In the presence of the oligonucleotide analyte in the sample, the ligation partner forms a double-stranded hybridization product with the analyte. A first strand of the hybridization product comprises the oligonucleotide analyte and the second strand of the

hybridization product, which is circular, comprises the ligation partner. The nucleotide sequence of the ligation partner is selected such that, when hybridized to the oligonucleotide analyte, forms a contiguous sequence of nucleotides with a nick between its ligation-accessible 3'-end and its ligation-accessible 5'-end.

The ligation partner(s) used according to the present invention are oligonucleotides having at least one sequence portion with a suitable length for hybridizing with a portion of the oligonucleotide analyte and an additional sequence portion, e.g. for hybridization with a complementary nucleotide sequence different from the oligonucleotide analyte, e.g. for hybridization with a primer.

In embodiments which involve the use of first and second ligation partners, the length of the respective oligonucleotides is usually at least 15 nucleotides and up to e.g. 200 nucleotides, particularly from about 25 to about 60 nucleotides. In embodiments which involve the use of a single ligation partner, its length is usually at least 40 nucleotides and up to e.g. 250 nucleotides, particularly about 50 to about 150 nucleotides.

The ligation partner(s) may comprise deoxyribonucleoside building blocks and optionally at least one modified nucleoside building block, particularly at least one locked nucleoside building block as described above. Further, the ligation partner (s) may comprise internucleosidic linkages selected from phosphodiester linkages and optionally at least one modified internucleosidic linkage such as a phosphorothioate linkage.

In a particular embodiment which involves the use of first and second ligation partners, the first ligation partner having a ligation-accessible 3'-end comprises at least one modified nucleoside building block, particularly at least one locked nucleoside building block, and the second ligation partner

having a ligation-accessible 5'-end does not comprise a modified nucleoside building block, particularly a locked nucleoside building block.

For example, the first ligation partner having a ligation-accessible 3'-end may comprise two modified nucleoside building blocks, particularly locked nucleoside building blocks. The modified nucleoside building blocks may be located in a sequence portion complementary to the nucleoside sequence of the oligonucleotide analyte. Further, the modified nucleoside building blocks may be separated by one or more non-modified nucleoside building blocks, particularly by four non-modified nucleoside building blocks.

In addition, the ligation partner(s) may comprise labelling and/or binding moieties. Examples of labelling moieties are e.g. fluorescent or luminescent groups. Examples of binding moieties are e.g. solid phase binding moieties which allow binding to a complementary binding partner which may be immobilized on solid phase, e.g. a bead, in particular a magnetic bead. An example of a suitable binding moiety is biotin which may be immobilized to a solid phase coated with streptavidin. The presence of binding moieties allows capture of reactants and separation from other sample constituents.

The test format of the method of the present invention may involve in some embodiments that at least steps (i) and (ii), or steps (i), (ii) and (iii) are carried out under conditions wherein the oligonucleotide analyte and the at least one ligation partners are in solution. In other embodiments, the method involves an immobilization of reactants on a solid phase and separation of non-immobilized sample constituents.

Step (iii) involves the closing of the nick between a ligation-accessible 3'-end and a ligation-accessible 5'-end of the at least one ligation partner by a ligation reaction, wherein a covalent bond is generated. The ligation-accessible 3'-end may be a free 3'-OH group at the 3'-end or a group which under test conditions may be converted to free 3'-OH group. The

ligation-accessible 5'-end is selected such that it can be ligated to the ligation-accessible 3'-end. Preferably, the ligation-accessible 5'-end comprises a phosphate group or a group which under test conditions may be converted to a phosphate group.

By means of the ligation reaction, a covalent bond, preferably a phosphodiester bond, is formed. The ligation reaction may be an enzymatic ligation catalyzed by a ligase enzyme capable of covalently joining a ligation-accessible 3'-end and a ligation-accessible 5'-end, e.g. a DNA ligase. Examples of suitable ligase enzymes are e.g. particularly selected from Taq DNA ligase, T4 DNA ligase, splint R ligase, *E. coli* DNA ligase, etc.

The ligation reaction is carried out in a suitable ligation buffer at a temperature at which the respective ligase is active and double-stranded hybrids of ligation partner(s) and oligonucleotide analyte may be formed, e.g. a temperature at about 25°C to 40°C, in particular about 38°C. In certain embodiments, the temperature may vary during the course of the ligation reaction. After ligation, the ligase is inactivated, e.g. by increasing the temperature to 50°C or higher, e.g. 65°C for a period of time which is sufficient to inactivate the respective ligase.

In certain aspects of the invention, at least one stacking partner of the oligonucleotide analyte is added to the sample before step (iii). The stacking partner is an oligonucleotide which hybridizes with the at least one ligation partner adjacent to the 3'-end or the 5'-end of the oligonucleotide analyte. In one embodiment, one stacking partner is added which hybridizes with the at least one ligation partner adjacent to the 3'-end of the oligonucleotide. In a further embodiment, one stacking partner is added which hybridizes with the at least one ligation partner adjacent to the 5'-end of the oligonucleotide analyte. In still a further embodiment, two stacking partners are added, one of which hybridizes adjacent to the 3'-end and the other one adjacent to the 5'-end of the oligonucleotide analyte.

When adding at least one stacking partner, the hybridization product of the oligonucleotide analyte and the at least one ligation partner generated in step (ii) additionally comprises the at least one stacking partner, i.e. a further oligonucleotide hybridized to the at least one ligation partner at a position adjacent to the 3'-end and/or the 5'-end of the oligonucleotide analyte. The stacking partner can form a contiguous sequence with the oligonucleotide analyte which is interrupted by a nick between the 3'-end of the stacking partner and the 5'-end of the oligonucleotide analyte or between the 5'-end of the stacking partner and the 3'-end of the oligonucleotide analyte.

The method of the invention does not involve a closing of the nick between the stacking partner and the oligonucleotide analyte in step (iii). This can be achieved by providing stacking partners which do not provide ligase-accessible combinations of 5'- and 3'-ends with the oligonucleotide analyte.

The stacking partner may be an oligonucleotide having a length of about 6 to about 50 nucleotides. It may optionally contain modified nucleoside building blocks, e.g. locked nucleoside building blocks and/or modified internucleosidic linkages, e.g. phosphorothioate linkages. The use of a stacking partner was found to increase the specificity and/or sensitivity of the analyte determination.

The stacking partners are selected such that they do not interfere with the subsequent amplification reaction using the ligation product as template. For example, the stacking partner may be a short oligonucleotide, e.g. with a length of about 6 to about 12 nucleotides, such that a double-stranded hybrid comprising the stacking partner with the ligation partner has a melting point ranking between the melting point of a hybrid of the oligonucleotide analyte and the ligation partner and the melting temperature of a hybrid of a primer used the subsequent amplification and the ligation partner. Alternatively, the stacking partner may be identical with one of the detection primers.

According to step (iv) of the method, the double-stranded hybridization product comprising oligonucleotide analyte and the at least one ligation partner is subjected to a nucleic acid amplification reaction. The amplification reaction is carried out under conditions which require the presence of the ligation product of step (iii) as a template. Thus, the amplification reaction will allow qualitative and quantitative determination of the oligonucleotide analyte.

In certain embodiments, the nucleic acid amplification reaction is real time quantitative PCR (qPCR) involving the use of primers binding to sequence portions of the at least one ligation partner which are not hybridized with the oligonucleotide analyte and carrying out successive amplification cycles involving primer elongation using a suitable DNA polymerase, in particular a thermostable DNA polymerase such as Taq DNA polymerase or variants thereof. The presence and/or amount of the ligation product generated in step (iii) correlates with the course of the amplification resulting in characteristic qPCR curves and quantification cycle (Cq) values.

The primers are oligonucleotides capable of hybridization with the ligation product generated by the ligation reaction in step (iii). In certain embodiments, the primers are selected such that they hybridize with sequence portions of the ligation partner(s) which are not complementary to the oligonucleotide analyte.

The method of the invention allows a relative or absolute quantitative determination of the oligonucleotide analyte in the sample based on the amount of ligation product present after step (iii). The reaction can be calibrated by using controls containing known amounts of the oligonucleotide analyte thereby allowing an absolute quantitative determination.

Other suitable methods for nucleic acid amplification comprise NASBA, rolling circle amplification, e.g. in embodiments where a single ligation

partner is used. These methods also allow a quantitative determination of the oligoanalyte based on the amount of ligation product initially present after step (iii) which correlates with the amount of oligonucleotide analyte in the sample.

In certain embodiments, the method of the invention can be carried out in a single combined reaction wherein the reactants of the ligation reaction, i.e. the at least one ligation partner and the ligase and optionally at least one stacking partner may be added to the sample at the same stage as the reactants of the nucleic acid amplification reaction, i.e. at least one primer, amplification enzyme, e.g. a DNA polymerase and nucleoside triphosphates. The inactivation of the ligase may then be the activation step of the nucleic acid amplification. Alternatively, the reactants of the nucleic acid amplification may be added to the sample after the ligation has been completed.

Further, the present invention relates to a kit for determining an oligonucleotide analyte, in particular an antisense oligonucleotide, in a sample. The kit optionally comprises the oligonucleotide analyte and at least one ligation partner adapted to the determination of the specific analyte as described above. Further, the kit may comprise optional components such as a ligase and/or at least one stacking partner and/or an instruction manual. Further, the kit may comprise the components used for the amplification reaction, in particular at least one primer, particularly a primer pair which hybridizes to the ligation product for use in the amplification reaction. The kit is particularly suitable for use in a method as described above.

Still a further aspect of the present invention relates to a method for directly determining an oligonucleotide analyte, particularly an antisense oligonucleotide, in a small nucleic acid, e.g. a RNA-containing fraction, such as a small RNA-containing fraction, of a biological sample, e.g. a micro-RNA-containing fraction which may be obtained with a suitable isolation protocol, e.g. the miRNeasy protocol from Qiagen. According to this aspect, the

oligonucleotide analyte may be determined without prior separation of other sample constituents, e.g. endogenous nucleic acid molecules present in the sample. The determination may be carried out according to any suitable method, e.g. methods involving ligation and/or amplification reactions. For example, the oligonucleotide analyte may be determined by combined ligation/amplification reaction, e.g. in a single combined reaction, particularly by the method as described above in detail.

Further, the present invention shall be explained by the following Figures and Examples.

Figure legends

Figure 1 shows an embodiment of the invention involving determination of an antisense oligonucleotide (ASO) with two ligation partner oligonucleotides 'a' and 'b'.

A: The ligation partner oligonucleotides 'a' and 'b' hybridize to the antisense oligonucleotide (ASO). In the double-stranded hybridization product, the ASO acts as a junction for bridging together the ligation partners 'a' and 'b' which form a contiguous sequence interrupted by a nick. The first (5') ligation partner 'a' has a ligation-accessible 3'-end, e.g. a 3'-OH group. The second (3') ligation partner 'b' has a ligation-accessible 5'-end which may be phosphorylated (P in circle) to enable ligation between the partners 'a' and 'b'. One of the ligation partners, e.g. the ligation partner 'a' can have a labelling or a binding group, e.g. a binding group to allow binding to a solid phase, e.g. a bead (B in circle).

B: After closing the nick between ligation partners 'a' and 'b' by means of a ligation reaction, the resulting ligation product is subjected to an amplification reaction. For example, the ligation product is used as a template in a PCR

reaction involving the addition of two suitable primers designated as FWD-DS and REV-DS.

Figure 2 shows the quantitative determination of an oligonucleotide analyte with 5'- and 3'-ligation partner oligonucleotides. The resulting number of quantification cycles (Cq) was found to be dependent from the amount of analyte present in the sample. The individual graphs are dilution curves based on Cq values for samples spiked with ASO GPanti-miR-205 in concentrations from 10 nM to 1 pM comparing ligations of a 5' partner (a) without LNA (blue and red curve) and a 5'-ligation partner (a) with LNA (purple and green curve), while the 3'-ligation partner contains either DNA (blue and green curves) or LNA (purple and red curve).

Figure 3 shows the difference in Cq values (ΔCQ) for reactions carried out in the presence or absence of a gapmer and a mixmer antisense oligo (ASO) using the different ligase enzymes. Different ligases ligate the substrate with different efficiencies. The difference of Cq values (ΔCQ) in the PCR ligation assay between 0 and 100 pM ASO anti-miR-205 (mixmer, blue) or a GPanti-miR-205 (gapmer, red) or a negative control m1-4 gapmer (NC, green) is depicted.

Figure 4 shows the detection of an ASO present in an RNA fraction isolated from cells after gymnotic transfection. qPCR Cq values (Y-Axis) are plotted against the gymnotic input amount of the ASO. NC negative control: absense of ASO. The ligation PCR assay can detect amounts of 0.001 to 1 µM ASO in a 1:100 dilution of the ligation reaction. The sample was total RNA directly isolated from cells. In the upper panel DNA ligation partners 'a' and 'b' were used, in the lower panel LNA ligation partners 'a' and 'b' were used.

Figure 5 shows a schematic overview of different embodiments wherein additional stacking partners are added prior to ligation.

A: Use of a reverse amplification primer as stacking partner for ligation partner b.

B: Use of 2 short oligonucleotides as stacking partners for ligation partners a and b. These oligos were designated as having a melting temperature ranging between the melting temperature of the ASO and the ligation partner and the melting temperature of the primer and the ligation partner.

Figure 6 shows the effect of the presence of stacking partners. Stacking partners enhance the detection sensitivity of the ASO. The change of Cq (ΔCQ) in the PCR ligation assay between 0 and 100 pM ASO m1-4 gapmer (blue) or an unrelated ASO GPanti-miR-205 (gapmer, red) is depicted. The use of the specifically designed stacking oligonucleotides (stacker a/b) or of the reverse primer (revp3opt) as a stacker preannealed to the corresponding ligation partner enhances the detection of the ASO.

Examples

Material and methods

Table 1: Oligonucleotides

anti-miR-205 (I)	C*C*+G*+G*+T*G*+G*+A*A*T*G*+A*A*+G*G	(SEQ ID NO: 1)
GPanti-miR-205 (II)	+C*+C*+G*G*T*G*G*A*A*T*G*+A*+A*+G*+G	(SEQ ID NO: 2)
m1-4gapmer (NC)	+C*+C*+A*A*T*G*C*T*A*G*T*/iMe-dC/*+G*+T	(SEQ ID NO: 3)
5a	GG*ACATGTTTCTGCGATGTC*CTATCCTTCATTC	(SEQ ID NO: 4)
5b	/5phos/CACCGGACA*GCTTGTTAGAACTACGCA*GT	(SEQ ID NO: 5)
5aLNA	GG*ACATGTTTCTGCGATGTC*CTAT+CCTT+CATTC	(SEQ ID NO: 6)
5bLNA	/5phos/CAC+CGG+ACA*GCTTGTTAGAACTACGCA*GT	(SEQ ID NO: 7)
FWD DS	AC+ATGTTTCTGCGATGTC	(SEQ ID NO: 8)
REV DS	T+GCGTAGTTCTAACAAGC	(SEQ ID NO: 9)
M1-4lig3a(-1)LNA	GGCAACGCTGTGTATCTCCCTATA+CGACT	(SEQ ID NO: 10)
M1-4lig3b(+1)	/5phos/AGCATTGGACAGAACTACTACAAATCTCCGGT	(SEQ ID NO: 11)
M1-4lig3b(+1)stk	/5phos/AGCATTGGGAACTACTACAAATCTCCGGT	(SEQ ID NO: 12)
stacker-a	+A+T+AG+GGA+G	(SEQ ID NO: 13)
stacker-b	+A+G+TT+C+T+G+T	(SEQ ID NO: 14)
M1-4 fwd p3opt	C+AACGCTGTGTATCTCC	(SEQ ID NO: 15)
M1-4 rev p3opt	C+GGAGA+TTTGTAGTAGTTC	(SEQ ID NO: 16)

Oligonucleotide sequences used in the experiments: + as prefix denotes LNA;* as suffix denotes phosphorothioate linkage, italics denote the primer binding sites for the PCR primers FWD DS and REV DS. The underlined sequences hybridize to ASO (I or II) but not to the control (NC). /5phos/ denotes a 5' phosphorylation of the oligonucleotide, /iMe-dC/ represents a 5 methyl dC base. Oligonucleotide concentrations below 1 µM were diluted in MS2 (50 ng MS2 in 80 µl H_2O).

Table 2: Enzymes and chemicals

Materials	Vendor	Cat No	Vendor (Example 4)	Cat No (Example 4)
MS2	Roche	10165948001		
Taq DNA ligase	NEB	M0208S		

T4 DNA ligase	NEB	M0202S	Enzymatics	L6030-LC-L
SplintR Ligase	NEB	M0375S		
E.coli DNA ligase	NEB	M0205S		
PEG 6000 50%	Sigma	81304		
PCR mastermix	Exiqon	203421	QIAGEN	2041423

Example 1

Introduction

To test the impact of LNA modified nucleotides within the ASO hybridization sequence of the ligation partner oligonucleotides on the assay readout we subjected four different combinations of ligation partners with or without LNA modification to ASO hybridization and ligation: 5a with 5b; 5a with 5bLNA; 5aLNA with 5b; 5aLNA with 5 bLNA. The ASO was added in 10 fold dilutions from 10 nM to 1 pM.

Assay

Ligase reaction

2 µl 5 oligo mix 1 nM

2 µl ASO II (GPanti-miR-205)

2 µl 10x buffer (Taq ligase)

2 µl Taq ligase

12 µl H_2O

The ligase reaction was run at 38°C for 120 min. Then the ligase was inactivated at 65°C for 10 min.

The ligation reaction was diluted 1:80 in the PCR reaction. Quantitative PCR was performed using SYBR green detection (Exilent mastermix, Exiqon) with 0.3 µM primer FWD DS and Rev-DS. The reaction conditions were 1 x 95°C for 10 min, 45 x 95°C for 10 sec, 60°C for 1 min, 1.6°C/sec, optical read, with melting curve analysis on a ROCHE LC480 instrument.

Conclusion

The data in Figure 2 show the effect on LNA modification of the ligation partners on the ligation and demonstrate that LNA modification in oligo 'a' increases sensitivity of the ligation PCR assay, whereas LNA modification in oligo 'b' decreases sensitivity of the ligation PCR assay under the chosen reaction conditions. The obtained Cq values reflect the input amount of ASO in a linear correlation and demonstrate that the assay allows to quantitatively determine ASO concentration through ligation and subsequent amplification by PCR.

Example 2

Introduction

Different ligases were tested for their efficiency to ligate the oligonucleotides 5a and 5b. Either ASO I anti-miR-205 with LNAs distributed throughout the sequence (mixmer) or ASO II GPanti-miR-205 with LNA at the wings and a central stretch of more than 5 unmodified nucleotides (gapmer) were used as junctions to hybridize the ligation partner oligonucleotides 5a and 5b.

Assay

Ligase reaction with different ligases all diluted in 1X reaction buffer (1:10)

2 µl oligo mix 1 nM (5a and 5b)

2 µl ASO I and II and NC 100 pM

2 µl 10x buffer (according to ligase)

2 µl ligase 1:10

4 µl PEG6000 (50%)

8 µl H_2O

The ligase reaction was run at 38°C for 120 min. Then the ligase was inactivated at 65°C for 10 min.

1 µl of the ligation reaction was used per 10 µl PCR reaction. Quantitative PCR was performed using SYBR green detection (2x Exilent Mastermix, Exiqon) with 0.3 µM primers FWD DS and Rev-DS. The reaction conditions were 1 x 95°C for 10 min, 45 x (95°C for 10 sec, 60°C for 1 min, 1.6°C/sec, optical read), with melting curve analysis on a ROCHE LC480 instrument.

Table 3: Results

1:10	(I)		(II)		NC		
Taq Ligase	19.32	19.23	18.61	18.82	31.47	30.64	100 pM
	30.4	30.33	31	31.73	31.06	31.17	No ASO
	42	34.16	34.38	33.8	40	34.11	No ligase
T4 DNA Ligase	16.13	16.26	15.4	15.59	29.88	30.21	100 pM
	28.94	29.08	29.62	29.82	29.82	30.35	No ASO
	25.54	25.67	33.04	33.5	35.12	40	No ligase
splintr Ligase	24.52	24.08	22.72	22.55	37.04	35.84	100 pM
	33.01	32.89	34.58	33.1	34.08	36.17	No ASO
	34.99	35.96	37.45	40	37.19	37.11	No ligase
E.coli DNA Ligase	30.1	30.02	24.5	24.91	36.75	34.76	100 pM
	33.94	40	34.05	32.33	35.02	34.58	No ASO
	33.24	32.96	31.52	31.92	34.01	38.24	No ligase

The ligation PCR assay detects both mixmer and gapmer ASO in an amount of 100 pM. The ligation partners can be ligated by different ligases. Cq values of anti-miR-205 (I), GPanti-miR-205 (II), m1-4gapmer (NC) are indicated. The controls "No ASO" were a ligation reaction in absence of ASO and "No ligase" a reaction in the absence of ligase and in the presence of 100 pM ASO.

Conclusion

The Cq differences between sample and negative control obtained with T4 DNA ligase and Taq DNA ligase show higher ligation efficiency than with splintR ligase or E. coli DNA ligase under the given conditions. Specificity is shown by the relatively high Cq difference and the lack of Cq change for the control ASO.

Example 3

Introduction

We tested whether the ASO quantification could be directly carried out with an RNA fraction isolated from different tissues or cell culture. We therefore tested in how far the ASO can be detected in RNA extracts from cells after gymnotic delivery of the ASO into the cells.

Assay

Hela cells were seeded in 24 well plates (1.38 $x10^5$ cells/well) and after 24 hrs subjected to gymnosis with 1 µM, 0.1 µM and 0.01 µM ASO, respectively, in DMEM media for 48 hrs. The cells were then washed twice with PBS and the RNA extracted according to the miRNEASY protocol (QIAGEN). The RNA was resuspended in 30 µl H_2O and 1 µl was subjected to ligation. The ligation mixture was diluted 1:100 and analysed via PCR.

Ligation:

2 µl oligo mix A or C (0.01 nM)
2 µl RNA from cells
2 µl 10 buffer (Taq ligase)
2 µl ligase 1:10
4 µl PEG 6000 50%
8 µl H_2O

The ligase reaction was run at 38°C for 120 min. Then the ligase was inactivated at 65°C for 10 min.

1 µl of the 1:100 diluted ligation reaction mixture per 10 µl PCR reaction was used. Quantitative PCR was performed using SYBR green detection (2 x Exilent Mastermix, Exiqon) with 0.3 µM primers FWD DS and Rev-DS. The reaction conditions were 1 x 95°C for 10 min, 45 x (95°C for 10 sec, 60°C for

1 min, 1.6°C/sec, optical read), with melting curve analysis on a ROCHE LC480 instrument.

Table 4: Results

1:100

ASO concentration	A (5a-5b) (I)antimiR		(II)GPantimiR		**m1-4gapmer**	
	I		II		NC	
	16.95	17.1	20.62	20.79	42	34.8
1 µM	16.93	16.88	21.7	21.98	35.79	35.17
	19.66	19.89	24.09	24.25	34.84	37.68
0,1 µM	19.33	19.79	25.76	26.02	33.88	33.8
	25.94	26.27	27.98	28.86	32.8	35.01
0,01 µM	24.22	24.63	28.03	28.62	33.98	35.38
	31.45	32.31	34.78	34.85	34.77	33.85
control	31.25	32.92	32.67	33.8	34.24	33.86

1:100

ASO concentration	C (5aLNA-5b) (I)antimiR		(II)GPantimiR		**m1-4gapmer**	
	I		II		NC	
	15.07	15.12	16.53	16.74	25.22	25.56
1 µM	14.75	14.92	15.08	14.97	26.71	26.52
	14.77	15	16.26	16.48	26.86	27.44
0,1 µM	13.85	14.96	16.91	17.18	26.8	26.72
	17.6	17.76	20.05	20.24	27.67	27.52
0,01 µM	16.5	16.8	19.72	19.91	25.98	25.87
	24.13	24.14	26.55	26.92	27.14	27.05
control	23.67	23.67	25.2	25.67	26.52	26.45

The ligation PCR assay detects mixmer and gapmer ASO from RNA isolated after gymnosis. The ligation partners can be LNA modified. The Cq values of anti-miR-205 (I), GPanti-miR-205 (II), m1-4gapmer (NC) for three final concentrations (1;0.1; 0.01 µM) are shown. The control was a reaction in the absence of ASO.

Conclusion

ASOs can be quantified directly from RNA isolated with the QIAGEN miRNEASY kit which preserves the small nucleic acid fraction. Thus no special lysis protocol is required. A linear correlation of Cq values with the amount of ASO allows quantification. The DNA ligation partners 5a and 5b showed linear correlation of Cq values with the input amount of ASO from 0.01 µM to 1 µM. The LNA ligation partner oligonucleotides 5a LNA – 5b showed saturation for gymnotic transfection with 1µM ASO under the given conditions.

Example 4

Introduction

To analyze a potential improvement of the ASO detection we prehybridized the ligation partners with different stacking oligonucleotides. These stacking oligonucleotides hybridize adjacent to the ASO binding part of the ligation partner. When all oligonucleotides are hybridized the stacking oligonucleotide and the ASO will form the first strand with a nick in between and the ligation partners form the second strand with a nick in between.

Assay

For the preeannealing the oligos (100 µM) were diluted 10 fold in annealing buffer (final conc. 10 mM Tris pH 7.5; 50 mM NaCl; 1 mM EDTA). Then, the oligonucleotides were heated to 95 °C for 2 min then cooled to 25 °C over 45 min and stored at 4 °C.

Depending on the design of the stacking oligonucleotides different partially double-stranded ligation partners will be obtained.

The pre-annealed ligation partners were further diluted to 100 pM and incubated with or without ASO in a touchdown ligation reaction:

oligo mix Ligation combinations	2 µl
ASO II and IV and NC	2 µl
10 Buffer (T4 ligase)	2 µl
ligase **1:10**	2 µl
PEG	4 µl
H_2O	8 µl
Total Ligase reaction	20 µl

The ligase reaction was carried out according to the following cycler program:

38°C	6,5'	1x		27°C	6,5'	1x
37°C	6,5'	1x		26°C	6,5'	1x
36°C	6,5'	1x		25°C	6,5'	1x
35°C	6,5'	1x		24°C	6,5'	1x
34°C	6,5'	1x		23°C	6,5'	1x
33°C	6,5'	1x		22°C	6,5'	1x
32°C	6,5'	1x		21°C	6,5'	1x
31°C	6,5'	1x		20°C	6,5'	1x
30°C	6,5'	1x		65°C	10'	1x
29°C	6,5'	1x		4°C	-	
28°C	6,5'	1x				

1 µl of the ligation reaction mixture per 10 µl PCR reaction was used. Quantitative PCR was performed using SYBR green detection (2x Quantitect SYBr green Mastermix, QIAGEN) with 0.3 µM primer M1-4 fwd p3opt and M1-4 rev p3opt. The reaction conditions were 1 x 95°C for 10 min, 45 x (95°C for 10 sec, 60°C for 1 min, 1.6°C/sec, optical read), with melting curve analysis on a ROCHE LC480 instrument.

Conclusion

The affinity of the ASO to hybridize to the ligation partner can be substantially enhanced through pre-hybridization of the ligation partner with a further stacking oligonucleotide.

Claims

1. A method for determining an oligonucleotide analyte in a sample, comprising

(i) adding to the sample at least one ligation partner of the oligonucleotide analyte,

wherein the at least one ligation partner is an oligonucleotide capable of hybridizing with the oligonucleotide analyte,

(ii) forming a hybridization product between the oligonucleotide analyte and the at least one ligation partner, wherein the oligonucleotide analyte bridges together two ends of the at least one ligation partner wherein a contiguous sequence of nucleotides interrupted by a nick between a ligation-accessible 3'-end and a ligation-accessible 5'-end of the at least one ligation partner is generated,

(iii) closing the nick by a ligation reaction, wherein a covalent bond, particularly a phosphodiester bond, between the accessible 3'-end and the accessible 5'-end of the at least one ligation partner is formed,

(iv) subjecting the ligation product to a nucleic acid amplification reaction, and

(v) qualitatively or quantitatively determining the product of amplification reaction,

wherein the oligonucleotide analyte is an antisense oligonucleotide, e.g. an antisense oligonucleotide comprising at

least one modified nucleoside building block, particularly a locked nucleoside building block, and/or at least one modified internucleosidic linkage, particularly a phosphorothioate linkage.

2. The method of claim 1, wherein the antisense oligonucleotide selected from the group of gapmers, blockmers, headmers, tailmers, mixmers and totalmers.

3. The method of claim 1 or 2, wherein the sample is a biological sample, e.g. a cell culture, tissue and/or body fluid sample.

4. The method of claim 3, wherein determination is carried out in an RNA-containing sample fraction.

5. The method of any one of claims 1-4, comprising adding to the sample a first ligation partner and a second ligation partner, wherein the first ligation partner comprises a 3'-terminal sequence portion with a ligation-accessible 3'-end which hybridizes with a 3'-terminal sequence portion of the oligonucleotide analyte and the second ligation partner comprises a 5'-terminal sequence portion with a ligation-accessible 5'-end which hybridizes with a 5'-terminal sequence portion of the oligonucleotide analyte thereby forming a linear contiguous sequence interrupted by a nick.

6. The method of any one of claims 1-4, comprising adding to the sample one ligation partner comprising a 3'-terminal sequence portion with a ligation-accessible 3'-end which hybridizes with a 3'-terminal sequence portion of the oligonucleotide analyte and a 5'-terminal sequence portion with a ligation-accessible 5'-end which hybridizes with a 5'-terminal sequence portion of the oligonucleotide analyte thereby forming a circular contiguous sequence interrupted by a nick.

7. The method of any one of claims 1-6, wherein the at least one ligation partner comprises at least one sequence portion for hybridization with a nucleotide sequence different from the oligonucleotide analyte, e.g. for hybridization with at least one primer.

8. The method of any one of claims 1-7, wherein the at least one ligation partner comprises at least one modified nucleotide building block, particularly a locked nucleotide building block and/or at least one modified internucleosidic linkage.

9. The method of any one of claims 1-8, wherein the ligation reaction is an enzymatic ligation catalyzed by a ligase, particularly by a DNA ligase, e.g. selected from Taq DNA ligase, T4 DNA ligase, splintR ligase or *E. coli* DNA ligase.

10. The method of claim 9, wherein the ligase is inactivated after step (iii).

11. The method of any one of claims 1-10, wherein at least one stacking partner of the oligonucleotide analyte is added to the sample before step (iii), wherein the stacking partner is an oligonucleotide which hybridizes with the at least one ligation partner adjacent to the 3'-end or the 5'-end of the oligonucleotide analyte.

12. The method of any one of claims 1-11, wherein the amplification and determination steps (iv) and (v) comprise real time quantitative PCR qPCR.

13. The method of any one of claims 1-12 which is carried out in a single combined reaction wherein the reactants of the ligation reaction may be added to the sample at the same stage as the reactants of the nucleic acid amplification reaction.

14. A kit for determining an oligonucleotide analyte in a sample comprising:

(a) optionally an oligonucleotide analyte,

(b) at least one ligation partner of the oligonucleotide analyte, wherein the oligonucleotide analyte is capable of hybridizing with the at least one ligation partner by forming a junction for bridging together two ends of the at least one ligation partner wherein a contiguous sequence of nucleotides interrupted by a nick between a ligation-accessible 3'-end and a ligation-accessible 5'-end of the at least one ligation partner is generated,

(c) optionally a ligase,

(d) optionally primers and/or stacking partners, and

(e) optionally an instruction manual.

Figure 1

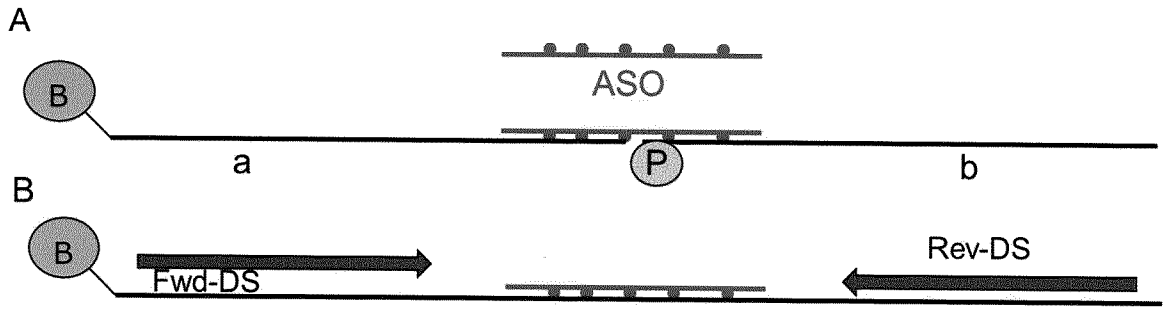
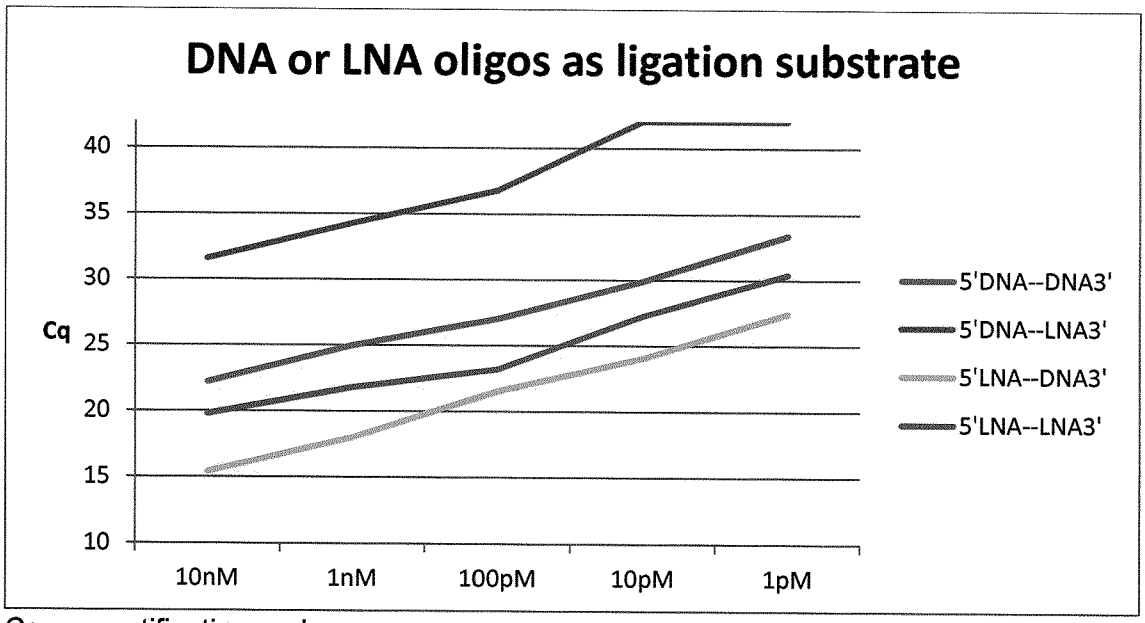


Figure 2



Cq = quantification cycle

Figure 3

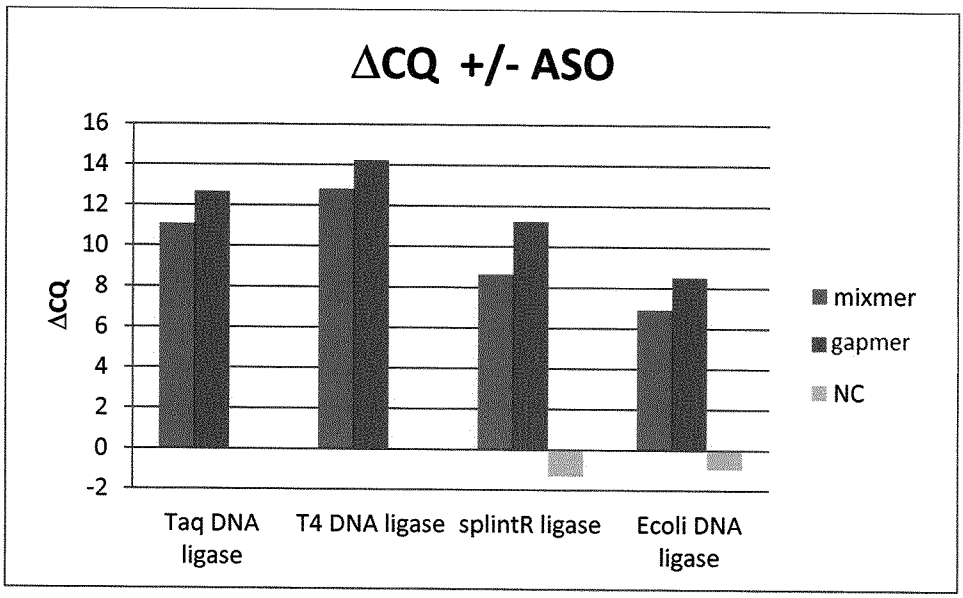
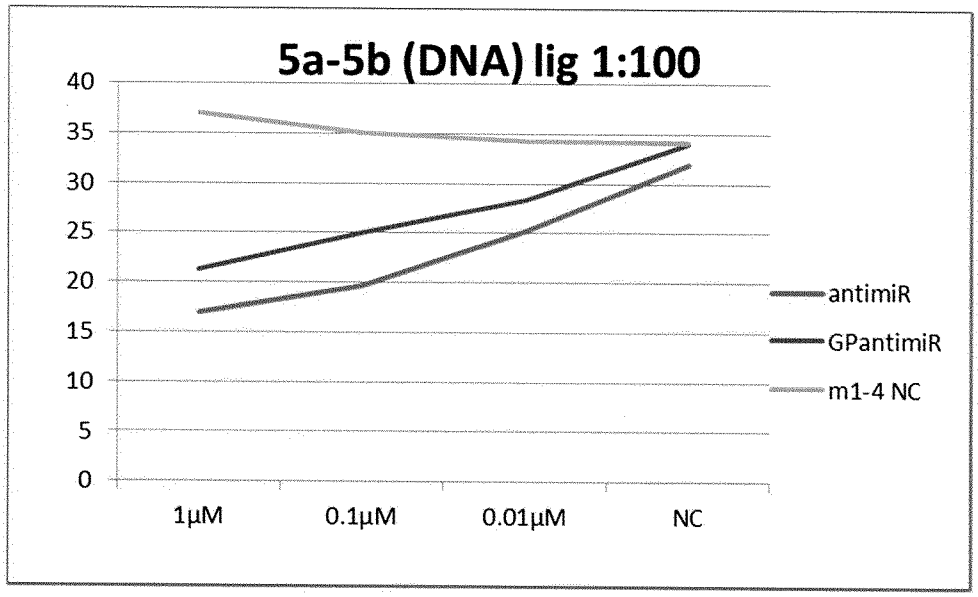

ΔCQ +/- ASO
ΔCQ
16
14
12
10
8
6
4
2
0
-2
Taq DNA ligase
T4 DNA ligase
splintR ligase
Ecoli DNA ligase
mixmer
gapmer
NC

Figure 4


5a-5b (DNA) lig 1:100
40
35
30
25
20
15
10
5
0
1µM
0.1µM
0.01µM
NC
antimiR
GPantimiR
m1-4 NC

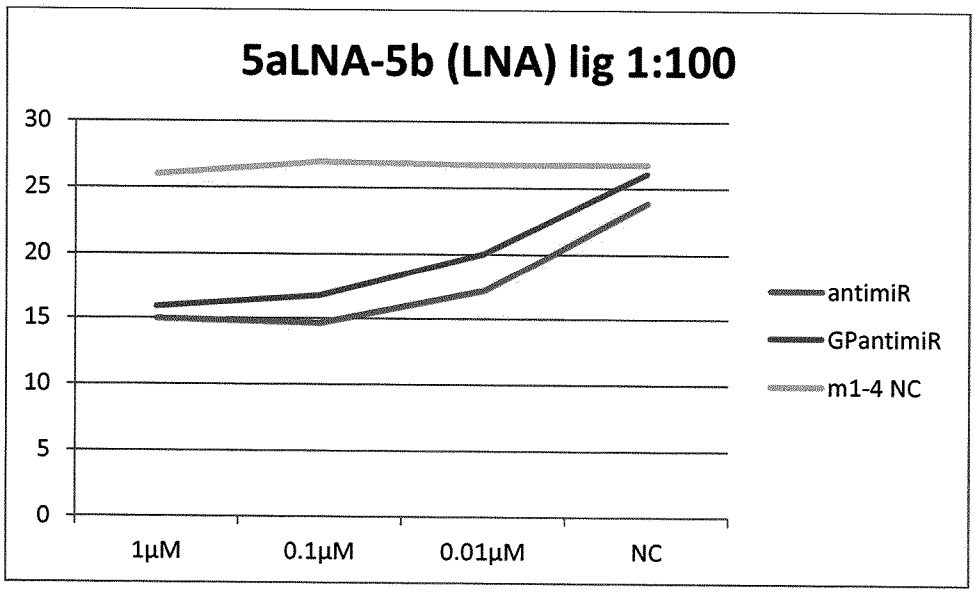
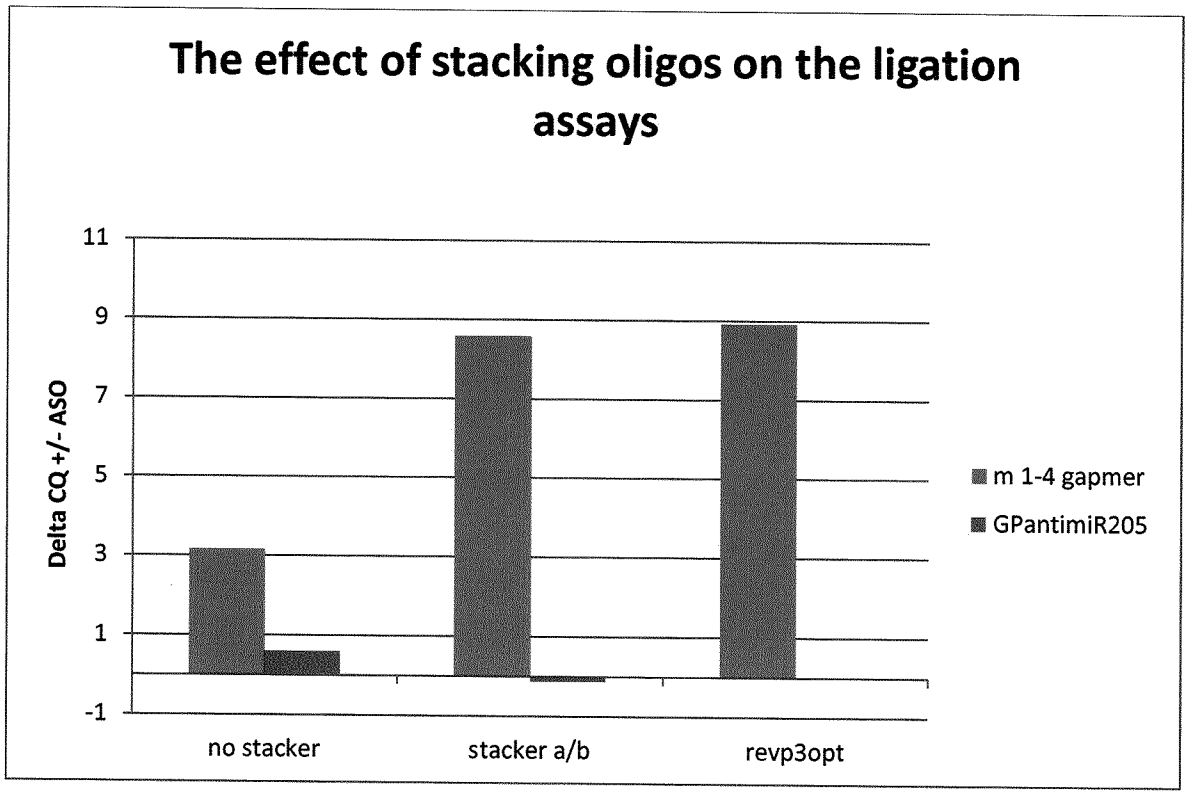

5aLNA-5b (LNA) lig 1:100
30
25
20
15
10
5
0
1µM
0.1µM
0.01µM
NC
antimiR
GPantimiR
m1-4 NC

Figure 5

A) use of the rev primer as a stacking partner

B) use of specifically designed stacking partners (LNA 8-mers with a Tm of ~45C)

Figure 6


The effect of stacking oligos on the ligation assays
Delta CQ +/- ASO
11
9
7
5
3
1
-1
no stacker
stacker a/b
revp3opt
m 1-4 gapmer
GPantimiR205

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/068572

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/085667 A2 (INTERNAT THERAPEUTICS INC [US]; KAWASAKI GLENN [US]; TRAVIS BRUCE [US]) 7 October 2004 (2004-10-07) page 2, line 17 - line 29; figure 1 page 4, paragraph 2 - paragraph 3 page 14, paragraph 3	1-14
X	WO 2005/098029 A2 (EXIQON AS [DK]; JACOBSEN NANA [DK]; KONGSBAK LARS [DK]; KAUPPINEN SAKA) 20 October 2005 (2005-10-20)	1-5,8-14
Y	figures 1,9 claims 32, 40 page 10, line 6 - line 7 page 68, paragraph 2	6

-/--

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

[X] See patent family annex.

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 August 2018	20/08/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schwachtgen, J

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/068572

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/04745 A1 (SINAI SCHOOL MEDICINE [US]) 5 February 1998 (1998-02-05)	14
Y	figures 1,2,4-7,14,15	6

X	WO 95/20679 A1 (HYBRIDON INC [US]) 3 August 1995 (1995-08-03) figure 1B	14

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/068572

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004085667 A2	07-10-2004	US 2004259128 A1	23-12-2004
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		CA 2180723 A1	03-08-1995
		EP 0734457 A1	02-10-1996
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		US 5637464 A	10-06-1997
		WO 9520679 A1	03-08-1995