

## **OLIGONUCLEOTIDES**

### **CROSS-REFERENCE TO RELATED APPLICATIONS**

The present application claims priority from Australian Provisional Patent  
5 Application No. 2020901606 filed on 19 May 2020, the contents of which are  
incorporated herein by reference in their entirety.

### **FIELD OF THE INVENTION**

The present invention relates to oligonucleotides that maintain a Toll-Like  
10 Receptor 7 (TLR7) response and/or which potentiate Toll-Like Receptor 8 (TLR8)  
sensing.

### **BACKGROUND OF THE INVENTION**

With the approval of eight oligonucleotides-based therapeutics in the US and  
15 European Union (Yin and Rogge, 2019; Al Shaer et al., 2020), and the prospect of many  
more to be commercialised from current phase III studies (Coutinho et al., 2019),  
therapeutic targeting of messenger RNA (mRNA) is set to play a large role in disease  
management. While different strategies have been developed to impact mRNA  
translation, such as recruiting RNase-H1 (with antisense oligonucleotides [ASOs] such  
20 as inotersen, or volanesorsen) or Ago2 (with small interfering RNAs [siRNAs] such as  
patisiran, inclisiran or givosiran) to actively degrade target mRNAs, or to promote  
splicing modulation (with ASOs such as eteplirsen and nusinersen), it is noteworthy that  
all therapeutic oligonucleotides approved to date rely on extensive chemical  
modifications. Such modifications are essential to prevent degradation by nucleases, and  
25 can also affect binding affinity to the target mRNA. These modifications can either be  
used to stabilise the phosphodiester (PO) internucleotide linkages, as seen with the  
phosphorothioate (PS) backbone modification, or to stabilise the bases with sugar  
modifications (e.g. with 2'-O-methyl [2'OMe], 2'- methoxyethyl [2'MOE], 2'-fluoro  
[2'F], or locked nucleic acid [LNA]) (Yin and Rogge, 2019).

30 In mammals, recognition of exogenous nucleic acids is a critical component of  
immune responses to pathogens and is achieved by a variety of innate immune sensors  
including, among others, the Toll-Like-Receptors (TLRs), such as TLR7, TLR8 and  
TLR9, the retinoic acid-inducible gene-I (RIG-I)-like receptors, NOD-like receptors, and  
the cyclic-GMP-AMP synthase (cGAS) pathway. It is therefore not surprising that select  
35 oligonucleotide therapeutics were found to instigate potent immune responses through  
direct engagement of such sensors (Hornung et al., 2005; Kleinman et al., 2008; Krieg et

al., 1995; Pichlmair et al., 2006), directing industry to closely consider and monitor such immune responses during pre-clinical and clinical development (Frazier et al., 2015). Nevertheless, discrimination between self and non-self nucleic acids by innate immune sensors can be modulated by the presence of nucleic acid modifications rarely encountered in pathogens – as seen with 2'-O-methylated (2'OMe) nucleosides that are 25 times more abundant in human ribosomal RNA than bacterial RNA (Kariko et al., 2005). TLR7 and TLR8 selectively detect RNA molecules and bases analogues (such as imidazoquinolines and nucleoside analogues), and are inhibited by 2'OMe bases, facilitating molecular discrimination between self and non-self RNAs (Kariko et al., 2005). As such, incorporation of select base modifications in therapeutic oligonucleotides, including 2'OMe, is a useful strategy to help mitigate aberrant immune responses by TLR7 and TLR8 (Kariko et al., 2005; Hamm et al., 2010), and is widely applied to therapeutic siRNAs (Coutinho et al., 2019).

However, this approach can also result in unintended immunosuppressive effects, as has been observed in the case of TLR7 and TLR8 antagonism by oligonucleotide sequences containing specific 2'OMe motifs (Sarvestani et al., 2015). Similarly, PS-modified DNA oligonucleotides have been reported to antagonise sensing by TLR9 (Gursel et al, 2003), TLR7 (Beignon et al, 2005), AIM2 (Kaminski et al., 2013) and cGAS (Steinhagen et al, 2018), in a sequence-dependent manner (Bayik et al., 2016). Critically, given that most therapeutic oligonucleotides currently approved or under investigation combine PS and base modifications, whether such combinations impact the frequency of immunosuppression is not currently defined.

Thus, there is a need for oligonucleotides with limited immunosuppressive effects on Toll-Like Receptor 7 (TLR7) and/or TLR8 responses.

#### **SUMMARY OF THE INVENTION**

While designing and testing oligonucleotides, the inventors observed structural features which assist in maintaining a Toll-Like Receptor 7 (TLR7) response.

Thus, in one aspect the present invention provides an oligonucleotide comprising three continuous pyrimidine bases within seven bases of the 5' and/or 3' end of the oligonucleotide.

In another aspect, the invention relates to an oligonucleotide comprising two continuous cytosine bases at or towards the 5' end of the oligonucleotide. Suitably, one or both of the two continuous cytosine bases are modified and/or which have a modified backbone.

In an embodiment, the oligonucleotide of the above aspects comprises

a) a 5' region comprising bases which are modified and/or which have a modified backbone,

b) a middle region comprising ribonucleic acid, deoxyribonucleic acid, or combination thereof, bases, and

5 c) a 3' region comprising bases which are modified and/or which have a modified backbone.

In a related aspect, the present invention provides an oligonucleotide comprising a 5' region, a 3' region and a middle region comprising ribonucleic acid, deoxyribonucleic acid, or combination thereof, bases, wherein one or both of the 5' region and the 3' region comprise bases which are modified and/or which have a modified backbone, and at least one of the following apply;

a) the 5' region comprises three continuous pyrimidine bases which are modified and/or which have a modified backbone,

b) the 5' region comprises bases which are modified and/or which have a modified backbone and the junction between the 5' region and middle region comprises three continuous pyrimidine bases,

c) the 3' region comprises three continuous pyrimidine bases which are modified and/or which have a modified backbone,

d) the 3' region comprises bases which are modified and/or which have a modified backbone and the junction between the 3' region and middle region comprises three continuous pyrimidine bases, and

e) the 5' region comprises two continuous cytosine bases which are modified and/or which have a modified backbone.

In an embodiment, the middle region is about 20, about 15 or about 10 bases in length.

In an embodiment, the 5' region and/or the 3' region are about 7, about 5, or about 3 bases in length.

In an embodiment, the three continuous pyrimidine bases are at or towards the 5' and/or 3' end of the oligonucleotide.

30 Examples of the 5' three continuous pyrimidine bases of the invention include, but are not limited to, those having the sequence 5'-CUU-3', 5'-CUT-3', 5'-CCU-3', 5'-UUC-3', 5'-UUU-3' or 5'-CTT-3'. In an embodiment, the 5' three continuous pyrimidine bases comprise the sequence 5'-CUU-3'.

35 Examples of the 3' three continuous pyrimidine bases of the invention include, but are not limited to, those having the sequence 5'-UUC-3', 5'-TUC-3', 5'-UCC-3', 5'-CUU-3', 5'-UUU-3' or 5'-TTC-3'. In an embodiment, the 3' three continuous

pyrimidine bases have the sequence 5'-UUC-3'. In another embodiment, the 3' pyrimidine bases have the sequence 5'-CUUC-3'.

In an embodiment, one, two or all three of the pyrimidine bases are a modified base and/or have a modified backbone.

- 5 In an embodiment, the three continuous pyrimidine bases at the junction have the sequence 5'-mCmUT-3', 5'-mCTT-3', 5'-TmUmC-3' or 5'-TTmC-3', where m is a modified base and/or has a modified backbone.

- Examples of modified bases useful for the invention include, but are not limited to, those which comprises a 2'-O-methyl, 2'-O-methoxyethoxy, 2'-fluoro, 2'-allyl, 2'-O-  
10 [2-(methylamino)-2-oxoethyl], 4'-thio, 4'-CH<sub>2</sub>-O-2'-bridge, 4'-(CH<sub>2</sub>)<sub>2</sub>-O-2'-bridge, 2'-LNA, 2'-amino, fluoroarabinonucleotide, threose nucleic acid or 2'-O--(N-methylcarbamate). In some embodiments, the modified base comprises a 2'-O-methyl, 2'-fluoro, 2'-allyl, 2'-O-[2-(methylamino)-2-oxoethyl], 4'-thio, 4'-CH<sub>2</sub>-O-2'-bridge, 4'-  
15 (CH<sub>2</sub>)<sub>2</sub>-O-2'-bridge, 2'-amino, fluoroarabinonucleotide, threose nucleic acid or 2'-O--(N-methylcarbamate).

- Examples of modified backbones useful for the invention include, but are not limited to, those which comprise a phosphorothioate, a non-bridging oxygen atom substituting a sulfur atom, a phosphonate such as a methylphosphonate, a phosphodiester, a phosphoromorpholidate, a phosphoropiperazidate, amides, methylene(methylamino),  
20 fromacetal, thioformacetal, a peptide nucleic acid or a phosphoroamidate such as a morpholino phosphorodiamidate (PMO), N<sup>3'</sup>-P<sup>5'</sup> phosphoramidite or thiophosphoramidite.

- In an embodiment, at least a portion of the oligonucleotide has/is a ribonucleic acid, deoxyribonucleic acid, DNA phosphorothioate, RNA phosphorothioate, 2'-O-  
25 methyl-oligonucleotide, 2'-O-methyl-oligodeoxyribonucleotide, 2'-O-hydrocarbyl ribonucleic acid, 2'-O-hydrocarbyl DNA, 2'-O-hydrocarbyl RNA phosphorothioate, 2'-O-hydrocarbyl DNA phosphorothioate, 2'-F-phosphorothioate, 2'-F-phosphodiester, 2'-methoxyethyl phosphorothioate, 2-methoxyethyl phosphodiester, deoxy methylene(methylimino) (deoxy MMI), 2'-O-hydrocarby MMI, deoxy-methylphosphonate, 2'-O-hydrocarbyl methylphosphonate, morpholino, 4'-thio DNA, 4'-thio RNA, 30 peptide nucleic acid, 3'-amidate, deoxy 3'-amidate, 2'-O-hydrocarbyl 3'-amidate, locked nucleic acid, cyclohexane nucleic acid, tricycle-DNA, 2'fluoro-arabino nucleic acid, N<sup>3'</sup>-P<sup>5'</sup> phosphoroamidate, carbamate linked, phosphotriester linked, a nylon backbone modification and any combination thereof.

- 35 In an embodiment, the modified base comprises a 2'-O-methyl and the oligonucleotide comprises a phosphorothioate backbone.

In some embodiments of the above aspects, the two continuous cytosine bases comprise a 2'-LNA and a phosphorothioate backbone.

In an embodiment, one, two or all three of the three continuous pyrimidine bases do not hybridize to a target polynucleotide.

5 In an embodiment of the above aspects, one or both of the two continuous cytosine bases do not hybridize to a target polynucleotide.

In another aspect, the present invention provides an oligonucleotide comprising

- i) 5'-CUUGU-3', 5'-CCUAU-3', 5'-CAUUA-3', 5'-CGAAU-3' 5'-CUUAU-3', 5'-CUUUA-3' or 5'-ACUGU-3' at the 5' end, and  
 10 ii) 5'-CUUCU-3' 5'-CAUAU-3' 5'-CUUCU-3' 5'-AAUUU-3' 5'-AAAUU-3' 5'-CCUUC-3', 5'-AAUCA-3' or 5'-CGUCU-3' at the 3' end.

In an embodiment of the above aspects, the oligonucleotide comprises a terminal 5'U. In another embodiment, the oligonucleotide comprises a terminal 5'UC.

In an embodiment, any one of the following is modified to comprise a 5' region,  
 15 preferably end, and/or a 3' region, preferably end, as described above;

5'-AUGGAAUACUCUUGGUUACTT-3' and/or 5'-GUAACCAAGAGUAUCCAUTT-3' (strands of the siRNA used to treat polyneuropathy referred to as Patisiran);

20 5'-GCGTTTGCTCTTCTTCTTGCG-3' (antisense oligonucleotide used to treat cytomegalovirus retinitis referred to as Fomivirsen);

5'-mG-mC\*-mC\*-mU\*-mC\*-dA-dG-dT-dC\*-dT-dG-dC\*-dT-dT-dC\*-mG-mC\*-mA-mC\*-mC\*-3', where m is a 2'-O-(2-methoxyethyl) nucleoside and d is a 2'-deoxynucleoside, with methyl group at position 5 of C and U (\*) (antisense oligonucleotide used to treat homozygous familial hypercholesterolemia referred to as

25 Mipomersen);

5'-MeU MeC MeU G GTTAMeCATGAAA MeU MeC MeC MeC-3', where underlined letters are 2'-O-(2-methoxyethyl)ribonucleotides; non-underlined letters are 2'-deoxyribonucleotides; all pyrimidines are 5-methylated; all linkages are phosphorothioates (antisense oligonucleotide used to treatment of nerve damage in adults  
 30 with hereditary transthyretin-mediated amyloidosis referred to as Inotersen);

5'-CUCCAACAUCAAGGAAGAUGGCAUUUCUAG-3' (antisense oligonucleotide used to treat Duchenne muscular dystrophy referred to as Eteplirsen);

5'-TCACTTTCATAATGCTGG-3' which is fully 2'-O-methoxyethyl (MOE) modified on a phosphorothioate backbone (antisense oligonucleotide used to treat spinal  
 35 muscular atrophy referred to as Nusinersen);

5'-XGTTGCCTCCGGTTCTGAAGGTGTTTC-3' where bases are linked through a synthetic neutral phosphorodiamidate morpholino oligomer (PMO) backbone and X is hydrophilic triethylene glycol chain (antisense oligonucleotide used to treat Duchenne muscular dystrophy referred to as Golodirsen); or

5' CAGAAAGAGUGUCUCAUCUUA 3' and/or 5' UAAGAUGAGACACUCUUUCUGGU 3' (strands of the siRNA used to treat acute hepatic porphyria referred to as Givosiran). Furthermore, such oligonucleotides may have other modifications such as those standard in the art.

In an embodiment, an oligonucleotide of any of the above aspects does not inhibit 10 Toll-like receptor 7 (TLR7) activity when administered to an animal. In an embodiment, the animal is a human.

In a further aspect, the present invention provides an oligonucleotide comprising one or more modified bases and at least four thymidines, wherein the oligonucleotide potentiates Toll-like receptor 8 (TLR8) activity when administered to an animal.

15 In an embodiment, the oligonucleotide comprises a 5'U. In another embodiment, the oligonucleotide comprises a 5'UC.

In an embodiment, the oligonucleotides comprises:

a) a 5' region at least five bases in length which are modified and/or which have a modified backbone.

20           b) a middle region comprising a stretch of ten bases, wherein at least four of the  
bases are thymidine,

c) a 3' region at least five bases in length.

In a further embodiment, the at least four thymidine bases are in a continuous stretch.

25 In another embodiment, one, two, three or four of the at least four thymidine bases  
are not in a continuous stretch.

In yet a further aspect, the present invention provides an oligonucleotide comprising

a) a 5' region at least five bases in length, wherein the 5' end consists of terminal

30 5'-mUmC-3' or terminal 5'-mCmU-3', where m is a modified base and/or has a modified backbone.

b) a middle region comprising a stretch of ten bases, wherein at least two of the bases are thymidine, and

c) a 3' region at least five bases in length and/or has a modified backbone,

35 wherein the oligonucleotide potentiates Toll-like receptor 8 (TLR8) activity when administered to an animal (such as a human).

In an embodiment, an oligonucleotide of the above two aspects is also an oligonucleotide as defined for the other aspects.

The oligonucleotide can be any size. Examples of suitable sizes include, but are not limited to at least about 10, at least about 18, at least about 20, at least about 21, at least about 22, at least about 23, at least about 24, at least about 25, at least about 26, at least about 27, at least about 28, at least about 29, at least about 30, at about least 40, between about 10 and about 50 nucleotides, between about 18 and about 50 nucleotides, between about 18 and about 30 nucleotides, between about 20 and about 30 nucleotides, between about 20 and 1,000 nucleotides, between about 20 and 5,000 nucleotides, or about 20 bases in length.

An oligonucleotide of the invention can be used for a variety of purposes. In one embodiment, the oligonucleotide is an antisense oligonucleotide such as for hybridizing to a target mRNA to reduce translation thereof. In another embodiment, the oligonucleotide is, or forms part of, a stranded oligonucleotide for gene silencing (such as RNA interference). In another embodiment, the oligonucleotide is used to potentiate Toll-like receptor 8 (TLR8) activity but does not hybridize to a target RNA.

In one embodiment, the oligonucleotide is a gapmer antisense oligonucleotide. In an embodiment, one, two or all three of the three continuous pyrimidine bases are removed by an endonuclease *in vivo*.

In an embodiment, the antisense oligonucleotide down regulates expression of a gene and potentiates Toll-like receptor 8 (TLR8) activity.

In an embodiment, the double stranded oligonucleotide for gene silencing is an siRNA or an shRNA.

In an embodiment, the oligonucleotide is between 10 and 16 bases in length and potentiates Toll-like receptor 8 (TLR8) activity when administered to an animal (such as a human).

In yet a further aspect, the present invention provides a method for selecting an oligonucleotide for reducing the expression of a target gene, the method comprising

i) scanning a target polynucleotide, or complement thereof, for a region with at least three continuous pyrimidine bases;

ii) producing one or more candidate oligonucleotides comprising the three continuous pyrimidine bases, wherein one or both of the following apply;

a) the candidate oligonucleotide comprises three continuous pyrimidine bases within seven bases of the 5' end of the oligonucleotide, and

b) the candidate oligonucleotide comprises three continuous pyrimidine bases within seven bases of the 3' end of the oligonucleotide,

iii) testing the ability of the one or more candidate oligonucleotides to reduce expression of the target gene, and

iv) selecting an oligonucleotide which reduces expression of the target gene.

In an embodiment, the three continuous pyrimidine bases of a candidate  
5 oligonucleotide have a modified base and/or a modified backbone.

In another aspect, the present invention provides a method for selecting an oligonucleotide for reducing the expression of a target gene, the method comprising

i) scanning a target polynucleotide, or complement thereof, for a region with at least three continuous pyrimidine bases;

10 ii) producing one or more candidate oligonucleotides comprising a 5' region, a 3' region and a middle region comprising ribonucleic acid, deoxyribonucleic acid, or combination thereof, bases, wherein one or both of the 5' region and the 3' region comprise bases which are modified and/or which have a modified backbone, and at least one of the following apply;

15 a) the 5' region comprises three continuous pyrimidine bases which are modified and/or which have a modified backbone,

b) the 5' region comprises bases which are modified and/or which have a modified backbone and the junction between the 5' region and middle region comprises three continuous pyrimidine bases,

20 c) the 3' region comprises three continuous pyrimidine bases which are modified and/or which have a modified backbone, and

d) the 3' region comprises bases which are modified and/or which have a modified backbone and the junction between the 3' region and middle region comprises three continuous pyrimidine bases,

25 iii) testing the ability of the one or more candidate oligonucleotides to reduce expression of the target gene, and

iv) selecting an oligonucleotide which reduces expression of the target gene.

In another aspect, the present invention provides a method for selecting an oligonucleotide for reducing the expression of a target gene, the method comprising

30 i) scanning a target polynucleotide, or complement thereof, for a region with one of the following sequences 5'-CUUGU-3', 5'-CCUAU-3', 5'-CAUUA-3', 5'-CGAAU-3' 5'-CUUAU-3', 5'-CUUUA-3', 5'-ACUGU-3', 5'-CUUCU-3' 5'-CAUAU-3' 5'-CUUCU-3' 5'-AAUUU-3' 5'-AAAUU-3' 5'-CCUUC-3', 5'-AAUCA-3' or 5'-CGUCU-3', wherein the U may be a T,

35 ii) producing one or more candidate oligonucleotides comprising

a) 5'-CUUGU-3', 5'-CCUAU-3', 5'-CAUUA-3', 5'-CGAAU-3' 5'-CUUUAU-3', 5'-CUUUA-3' or 5'-ACUGU-3' at the 5' end, and/or

b) 5'-CUUCU-3' 5'-CAUAU-3' 5'-CUUCU-3' 5'-AAUUU-3' 5'-AAAUU-3' 5'-CCUUC-3', 5'-AAUCA-3' or 5'-CGUCU-3' at the 3' end,

5       iii) testing the ability of the one or more candidate oligonucleotides to reduce expression of the target gene, and

iv) selecting an oligonucleotide which reduces expression of the target gene.

In still a further aspect, the present invention resides in a method for selecting an oligonucleotide for reducing the expression of a target gene, the method comprising

10       i) scanning a target polynucleotide, or complement thereof, for a region with at least two continuous cytosine bases;

ii) producing one or more candidate oligonucleotides comprising the two continuous cytosine bases, wherein the candidate oligonucleotide comprises two continuous cytosine bases at or towards the 5' end of the oligonucleotide,

15       iii) testing the ability of the one or more candidate oligonucleotides to reduce expression of the target gene, and

iv) selecting an oligonucleotide which reduces expression of the target gene.

In some embodiments, the two continuous cytosine bases of the oligonucleotide have a modified base and/or a modified backbone.

20       In other embodiments, the oligonucleotide comprises:

a) a 5' region comprising bases which are modified and/or which have a modified backbone,

b) a middle region comprising ribonucleic acid, deoxyribonucleic acid, or combination thereof, bases, and

25       c) a 3' region comprising bases which are modified and/or which have a modified backbone.

In one embodiment, the 5' region and/or the 3' region are about 3 bases in length.

In another embodiment, the middle region is about 10 bases in length.

In a related aspect, the invention relates to a method for selecting an oligonucleotide for reducing the expression of a target gene, the method comprising

30       i) scanning a target polynucleotide, or complement thereof, for a region with at least two continuous cytosine bases;

ii) producing one or more candidate oligonucleotides comprising a 5' region, a 3' region and a middle region comprising ribonucleic acid, deoxyribonucleic acid, or

35       combination thereof, bases, wherein one or both of the 5' region and the 3' region comprise bases which are modified and/or which have a modified backbone, and the 5'

region comprises two continuous cytosine bases which are modified and/or which have a modified backbone,

iii) testing the ability of the one or more candidate oligonucleotides to reduce expression of the target gene, and

5 iv) selecting an oligonucleotide which reduces expression of the target gene.

In one embodiment, the 5' region and/or the 3' region are about 3 bases in length.

In another embodiment, the middle region is about 10 bases in length.

In certain embodiments of the above two aspects, one or both of the two continuous cytosine bases are a modified base and/or have a modified backbone.

10 Suitably, for the two above aspects, the two continuous cytosine bases comprise a 2'-LNA and a phosphorothioate backbone.

In an embodiment of the three above aspects, the method further comprises testing the ability of the one or more candidate oligonucleotides to inhibit Toll-like receptor 7 (TLR7) activity, and selecting an oligonucleotide which does not inhibit TLR7  
15 activity. In this regard, the methods of the above aspects are suitably for decreasing the TLR7 inhibitory activity of the oligonucleotide.

While designing and testing oligonucleotides, the inventors also observed new structural features which assist in potentiating Toll-like receptor 8 (TLR8) activity.

Thus, in yet a further aspect, the present invention provides a method for selecting  
20 an oligonucleotide for reducing the expression of a target gene, the method comprising

i) scanning a target polynucleotide, or complement thereof, for a region comprising at least four of the bases are thymidine;

ii) producing one or more candidate oligonucleotides comprising one or more modified bases and at least four thymidines,

25 iii) testing the ability of the one or more candidate oligonucleotides to reduce expression of the target gene and to potentiate Toll-like receptor 8 (TLR8) activity, and

iv) selecting an oligonucleotide which reduces expression of the target gene and which potentiates TLR8 activity.

In another aspect, the present invention provides a method for selecting an  
30 oligonucleotide which potentiates Toll-like receptor 8 (TLR8) activity, the method comprising

i) scanning a target polynucleotide, or complement thereof, for a region with the sequence UC or CU and a stretch of ten bases, wherein at least two of the bases are thymidine;

35 ii) producing one or more candidate oligonucleotides comprising;

a) a 5' region at least five bases in length, wherein the 5' end consists of terminal 5'-mUmC-3' or terminal 5'-mCmU-3', where m is a modified base and/or has a modified backbone,

b) a middle region comprising a stretch of ten bases, wherein at least two  
5 of the bases are thymidine, and

c) a 3' region at least five bases in length and/or has a modified backbone,  
iii) testing the ability of the one or more candidate oligonucleotides to potentiate  
TLR8 activity, and

iv) selecting an oligonucleotide which potentiates TLR8 activity.

10 In some instances it may not be possible to design a suitable oligonucleotide with the required pyrimidine bases. Alternatively, in other instances it may be desirable to improve the functioning of a pre-existing oligonucleotide which lacks the required pyrimidine bases. Thus, in another aspect, the present invention provides a method of reducing the Toll-like receptor 7 (TLR7) inhibitory activity of an oligonucleotide, the  
15 method comprising modifying the oligonucleotide by adding a sequence of nucleotides to the 5' and/or 3' end of the oligonucleotide such that the modified oligonucleotide comprises three continuous pyrimidine bases within seven bases of the 5' and/or 3' end of the oligonucleotide.

In an embodiment, one, two or all three of the pyrimidine bases are a modified  
20 base and/or have a modified backbone.

In another aspect, the present invention provides a method of reducing the Toll-like receptor 7 (TLR7) inhibitory activity of an oligonucleotide, the method comprising modifying the oligonucleotide such that the modified oligonucleotide comprises at least one of the following;

25 a) the 5' region comprises three continuous pyrimidine bases which are modified and/or which have a modified backbone,

b) the 5' region comprises bases which are modified and/or which have a modified backbone and the junction between the 5' region and middle region comprises three continuous pyrimidine bases,

30 c) the 3' region comprises three continuous pyrimidine bases which are modified and/or which have a modified backbone, and

d) the 3' region comprises bases which are modified and/or which have a modified backbone and the junction between the 3' region and middle region comprises three continuous pyrimidine bases.

35 In an embodiment of the two above aspects, the three continuous pyrimidine bases are at the 5' and/or 3' end of the modified oligonucleotide.

In a further aspect, the invention provides a method of reducing the Toll-like receptor 7 (TLR7) inhibitory activity of an oligonucleotide, the method comprising modifying the oligonucleotide by adding a sequence of nucleotides to the 5' end of the oligonucleotide such that the modified oligonucleotide comprises two continuous  
5 cytosine bases at or towards the 5' end of the oligonucleotide.

In certain embodiments, one or both of the two continuous cytosine bases are a modified base and/or have a modified backbone.

In a related aspect, the invention resides in a method of reducing the Toll-like receptor 7 (TLR7) inhibitory activity of an oligonucleotide, the method comprising  
10 modifying the oligonucleotide such that the modified oligonucleotide comprises a 5' region comprising two continuous cytosine bases which are modified and/or which have a modified backbone.

In particular embodiments of the above two aspects, the two continuous cytosine bases are at or towards the 5' end of the modified oligonucleotide.

15 In other embodiments of the above two aspects, the two continuous cytosine bases comprise a 2'-LNA and a phosphorothioate backbone.

In another embodiment of the four above aspects, the method further comprises testing the ability of the modified oligonucleotide to inhibit TLR7 activity, and selecting an oligonucleotide which inhibits (TLR7) activity to a lesser extent than the unmodified  
20 oligonucleotide.

Also provided is an oligonucleotide selected using the method of the invention, or modified using a method of the invention.

In another aspect, the present invention resides in an oligonucleotide comprising, consisting of or consisting essentially of a nucleic acid sequence set forth in Tables 1 to  
25 6 or a variant thereof.

In another aspect, the present invention provides a composition comprising an oligonucleotide of the invention.

In an embodiment, the composition further comprises a pharmaceutically acceptable carrier.

30 In another embodiment, the composition further comprises an immune response modifier.

In another aspect, the present invention provides a method of reducing expression of a target gene in a cell, the method comprising contacting the cell with an oligonucleotide of the invention.

35 In another aspect, the present invention provides a method of treating or preventing a disease in a subject, the method comprising administering to the subject an

oligonucleotide of the invention, wherein the oligonucleotide reduces the expression of a target gene involved in the disease.

In an embodiment, the animal has been, or will be, administered with an immune response modifier.

5 In an embodiment, the immune response modifier is a Toll-like receptor (TLR) agonist. Examples of suitable Toll-like receptor (TLR) agonists include, but are not limited to, a base analogue (including: a guanosine analogue, a deaza-adenosine analogue, an imidazoquinoline or a derivative, a hydroxyadenine compound or a derivative, a thiazoloquinolone compound or a derivative, a benzoazepine compound or  
10 a derivative), or an RNA molecule.

In an embodiment, the TLR agonist is Guanosine, Uridine, Resiquimod (R848), Loxoribine, Isatoribine, Imiquimod, CL075, CL097, CL264, CL307, 852A, or TL8-506.

Also provided is the use of an oligonucleotide of the invention in the manufacture of a medicament for treating or preventing a disease in a subject, wherein the  
15 oligonucleotide reduces the expression of a target gene involved in the disease.

Further, provided is an oligonucleotide of the invention for use in treating or preventing a disease in a subject, wherein the oligonucleotide reduces the expression of a target gene involved in the disease.

Any embodiment herein shall be taken to apply *mutatis mutandis* to any other  
20 embodiment unless specifically stated otherwise.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

25 Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or group of compositions of matter.

30 The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

## 35 **BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS**

**Figure 1.** ASO-dependent modulation of R848 sensing by TLR7/8.

- (A) Wild-type THP-1 pre-treated overnight with 100 nM indicated ASO targeted to cGAS (Table 1), were stimulated or not (non-treated [NT]) with 1 µg/ml R848 for 8.5 h, and IP-10 (left panel) and TNF-α (right panel) levels in supernatants determined by ELISA. Data shown are averaged from three (left) or two (right) independent experiments in biological triplicate (± s.e.m and ordinary one-way ANOVA with Dunnett's multiple comparison tests to the "R848 without ASO" condition are shown). There was no basal effect of the ASOs on NT cells for either cytokines.
- (B, C) HEK-TLR7 (B) and HEK-TLR8 (C) cells expressing an NF-κB-luciferase reporter were treated with 500 nM indicated ASOs for 50 min prior to stimulation with 1 µg/ml R848. NF-κB-luciferase levels were measured after overnight incubation. Percentages (B) or fold increases (C) relative to the condition "R848 without ASO" condition are averaged from three independent experiments in biological triplicate (± s.e.m and ordinary one-way ANOVA with Dunnett's multiple comparison tests to the NT condition [B] or the "R848 without ASO" condition [C] are shown).
- (D) UNC93B1-deficient THP-1 (KO) and matched controls with rescued UNC93B1 expression (WT) were treated with 100 nM ASO overnight, prior to stimulation with 1 µg/ml R848 for 24 h and IP-10 levels in supernatants determined by ELISA. Data shown are averaged from two independent experiments in biological triplicate for each cell line (± s.e.m and unpaired t-tests are shown). The cGAS ligand ISD70 was used at 2.5 µg/ml as positive control to induce IP-10. (E, F) PBMCs from two blood donors were incubated 20-45 min with 100 nM ASO, and stimulated with 0.5 µg/ml R848 for 4 h prior to TNF-α ELISA (E), or 24 h prior to IFN-α ELISA (F).
- (E) Data shown are averaged from 2 blood donors in biological triplicate (± s.e.m. and ordinary one-way ANOVA with Dunnett's multiple comparison tests to the "R848 without ASO" condition are shown).
- (F) Data were normalised to the condition "R848 without ASO" to limit variations between patients, and are averaged from 2 blood donors in biological triplicate (± s.e.m. and Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparison tests to the "R848 without ASO" condition are shown).
- (G) [cGAS]ASO2 sequence variants used. The central DNA region is highlighted in light grey. For the 3' and 5' flanking regions (highlighted in dark grey), the DNA bases are in black, the five 5' and 3' bases of ASO2, ASOs-Cys3 and ASO-PO are 2'OMe bases, the three 5' and 3' bases of ASO2-LNA are LNA bases and the five 5' and 3' bases of ASO2-MOE are 2'MOE bases. Underlined bases are on a PS backbone.
- (H, I) HEKTLR7 (H) and HEK-TLR8 (I) cells expressing an NF-κB-luciferase reporter were treated with 500 nM indicated ASOs for 20 min prior to stimulation with 1 µg/ml

R848. NF- $\kappa$ B-luciferase levels were measured after overnight incubation. Percentages (H) or fold increases (I) relative to the condition “R848 without ASO” condition are averaged from three (I) or two (H) independent experiments in biological triplicate ( $\pm$  s.e.m and ordinary one-way ANOVA with Dunnett’s multiple comparison tests to the NT condition [H] or the “R848 without ASO” condition [I] are shown). \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , \*\*\*\*  $P \leq 0.0001$ , ns: non-significant.

**Figure 2.** Identification of ASOs with low TLR7 inhibition and high TLR8 potentiation. (A, B) HEK-TLR7 (A) and HEK-TLR8 (B) cells expressing an NF- $\kappa$ B-luciferase reporter were treated with 100 nM or 500 nM indicated ASOs for 20-50 min prior to stimulation with 1  $\mu$ g/ml R848. NF- $\kappa$ B-luciferase levels were measured after overnight incubation. Percentages (A) or fold increases (B) relative to the condition “R848 without ASO” are averaged from biological duplicates (averaged data are provided in Table 2). Stimulations with 100 nM and 500 nM ASO were performed in independent experiments (data shown for each concentration is from a single experiment). [CDKN2B-AS1]-852, and [LINCPINT]-2504 are referred to as ASO852 and ASO2504, and are indicated on the plot. ASOs with  $\geq 80\%$  reduction of TLR7 activity at 500 nM (A) and  $\leq 2$  fold TLR8 potentiation at 100 nM (B) are highlighted with grey shading.

(C, D) HEK-TLR7 (left panels) and HEK-TLR8 (right panels) cells expressing an NF- $\kappa$ B-luciferase reporter were treated with increasing ASOs concentrations (4, 20, 100 and 500 nM) (C) or with 500 nM ASOs (D) for 20 min prior to stimulation with 1  $\mu$ g/ml R848 (C) or with increasing R848 concentrations (0.0156, 0.031, 0.062 0.125, 0.250, 0.5, 1  $\mu$ g/ml) (D). NF- $\kappa$ B-luciferase levels were measured after overnight incubation. Percentages (left panels) or fold increases (right panels) relative to the condition “R848 without ASO” (C) or NT condition (D) are averaged from two independent experiments in biological triplicate ( $\pm$  s.e.m and ordinary two-way ANOVA with Dunnett’s multiple comparison tests to the ASO4 condition [C] or R848 only condition [D] are shown). \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , \*\*\*\*  $P \leq 0.0001$ , ns: non-significant.

**Figure 3.** Identification of molecular determinants of ASO effect on TLR7/8. (A) Top: sequence alignments of ASOs from the screens on HEK-TLR7 cells (Fig. 2A) which displayed low TLR7 inhibition at 100 nM and harboured significantly enriched motif (see Table 2 for detail of the 17 ASOs used in this analysis). [CD.] is CDKN2B-AS1; [CT.] is CTNNB1. The central DNA region is highlighted in light grey and the 3’ and 5’ 2’OMe flanking regions are highlighted in dark grey. Bottom: MEME pictogram

of the relative frequency of bases constituting the non-inhibitory motif. CUU motifs are in bold light grey, while UUC motifs are underlined.

(B, E) HEK-TLR7 cells expressing an NF- $\kappa$ B luciferase reporter were treated with 100 nM (B) or 500 nM (E) indicated ASOs for 20 min prior to stimulation with 1  $\mu$ g/ml R848.

5 NF- $\kappa$ B-luciferase levels were measured after overnight incubation. Percentages relative to the condition “R848 without ASO” are averaged from three (B) or two (E) independent experiments in biological triplicate ( $\pm$  s.e.m and ordinary one-way ANOVA with Dunnett’s multiple comparison tests to the NT condition [B] or Mann-Whitney U tests [E] are shown).

10 (C, D) Sequence alignments of ASOs from the PINT series (C) and [cGAS]ASO11 variants (D). (C) The conserved region between all the sequences is highlighted in light grey. The 2’OMe flanking regions are highlighted in dark grey. (D) The central DNA region is highlighted in light grey and the 3’ and 5’ 2’OMe flanking regions are highlighted in dark grey. (C, D) CUU motifs are in bold light grey, while UUC motifs are underlined.

(F, G) HEK-TLR8 cells expressing an NF- $\kappa$ B-luciferase reporter were treated with 100 nM (F) or 500 nM (G) indicated ASOs for 20 min prior to stimulation with 1  $\mu$ g/ml R848. NF- $\kappa$ B-luciferase levels were measured after overnight incubation. Fold increases relative to the condition “R848 without ASO” are averaged from three (F) or two (G)

20 independent experiments in biological triplicate ( $\pm$  s.e.m and ordinary one-way ANOVA with Dunnett’s multiple comparison tests to the NT condition [F] or Mann-Whitney U tests [G] are shown).

(H, I) 192 ASOs from the screen were sorted into two groups according to presence/absence of terminal 5’mU (H) or 5’mUmC (I), and fold increase NF- $\kappa$ B-luciferase levels to R848 only (using 500 nM ASOs – Table 2) are shown as violin plots for each population. Mann-Whitney U tests are shown.

(J) The central 10 DNA bases of the top and bottom 20 TLR8 potentiators from the 192 ASOs screened (Table 2) was analysed for base content. The violin plots show the distribution of the cumulative number of each central base for both ASO populations.

30 Ordinary two-way ANOVA with Sidak’s multiple comparison tests are shown.

(K) ASO852 and ASO2504 variants. The central DNA region is highlighted in blue and the 3’ and 5’ 2’OMe flanking regions are highlighted in orange.

(L) WT THP-1 were pre-treated overnight with 100 nM ASO, and stimulated with 1  $\mu$ g/ml R848 for 7 h and IP-10 levels in supernatants determined by ELISA. Data shown are averaged from two independent experiments in biological triplicate ( $\pm$  s.e.m and unpaired t-tests are shown).

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(M) HEK-TLR7 and HEK-TLR8 cells expressing an NF- $\kappa$ B-luciferase reporter were treated with 500 nM indicated ASOs for 20 min prior to stimulation with 1  $\mu$ g/ml R848. NF- $\kappa$ B-luciferase levels were measured after overnight incubation. Percentages or fold increases relative to the condition “R848 without ASO” condition are averaged from three independent experiments in biological triplicate ( $\pm$  s.e.m and ordinary one-way ANOVA with Dunnett’s multiple comparison tests to the NT condition [TLR7] or Mann-Whitney U test [TLR8] are shown). \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , \*\*\*\*  $P \leq 0.0001$ , ns: non-significant.

- 10 **Figure 4.** Characterization of ASOs potentiation of R848 sensing by TLR8.
- (A) MOLM13 and OCIAML3 cells were incubated overnight with 500 nM ASOs, stimulated with 1  $\mu$ g/ml R848 for 8 h (MOLM13) or 24 h (OCI-AML3), and IP-10 levels in supernatants determined by ELISA. Data shown are averaged from five (MOML13) or four (OCI-AML3) independent experiments for all conditions with exception of the
- 15 ASO only conditions (carried out in two independent experiments) in biological triplicate ( $\pm$  s.e.m and ordinary one-way ANOVA with Dunnett’s multiple comparison tests to the “R848 without ASO” condition are shown).
- (B) MOLM13 and THP-1 were incubated overnight with increasing doses of ASOs (4, 20, 100, 500 nM), stimulated with 1  $\mu$ g/ml R848 for 8 h and IP-10 levels in supernatants
- 20 determined by ELISA. Data shown are averaged from two independent experiments in biological triplicate ( $\pm$  s.e.m and ordinary two-way ANOVA with Dunnett’s multiple comparison tests to the ASO4 condition are shown).
- (C) HEK-TLR8 cells expressing a CCL5-luciferase reporter (right-hand side) or an IFN- $\beta$ -luciferase reporter (left-hand side) were treated with 500 nM indicated ASOs for 20
- 25 min prior to stimulation with 1  $\mu$ g/ml R848. Luciferase levels were measured after overnight incubation. Data are shown as fold increase to NT condition, and are averaged from two independent experiments in biological triplicate ( $\pm$  s.e.m and ordinary one-way ANOVA with Dunnett’s multiple comparison tests to the NT condition are shown).
- (D) WT THP-1 were incubated overnight with 500 nM ASO852-dT (or NT), and
- 30 stimulated or not for 4 h with 1  $\mu$ g/ml R848, prior to RNA purification. Expression of panel of 4 IRF-driven genes was analysed by RT-qPCR. Expression of the indicated genes was reported to 18S expression, and further normalised to the average of the “R848 without ASO” condition. Data shown represent the average of two independent experiments conducted in biological duplicate ( $\pm$  s.e.m and MannWhitney U tests are
- 35 shown).

(E) HEK-TLR8 cells expressing an IFN- $\beta$ -luciferase reporter were treated with 500 nM ASO852-dT (or NT) for 20 min prior to stimulation with increasing doses of R848 (1, 5, 10, 15  $\mu$ g/ml). IFN- $\beta$ -luciferase levels were measured after overnight incubation. Data are shown as fold increase to NT condition, and are averaged from two independent experiments in biological triplicate ( $\pm$  s.e.m and ordinary one-way ANOVA with Tukey's multiple comparison tests to the NT condition and selected pairs of conditions are shown). \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , \*\*\*\*  $P \leq 0.0001$ , ns: non-significant.

**Figure 5.** Rational selection of HPRT-targeting ASOs exhibiting TLR8 potentiation.

- 10 (A) HeLa cells were reverse-transfected with 1nM (left-hand side) or 10nM of (right-hand side) HPRT-targeting ASOs as detailed in Example 1 and Table 3, and HPRT levels measured by RT-qPCR after 24h incubation. HPRT levels were reported to SFRS9 expression, and further normalised to the average of the non-targeting ASONC1 and ASONC5 control conditions. Data shown represent the average of three independent experiments conducted in biological triplicate ( $\pm$  s.e.m).
- (B, C) HEKTLR7 (B) and HEK-TLR8 (C) cells expressing an NF- $\kappa$ B-luciferase reporter were treated with 500 nM ASOs for 20 min prior to stimulation with 1  $\mu$ g/ml R848. NF- $\kappa$ B-luciferase levels were measured after overnight incubation. Percentages (B) or fold increases (C) relative to the condition "R848 without ASO" (B) or NT condition (C) are averaged from three independent experiments in biological triplicate ( $\pm$  s.e.m and ordinary one-way ANOVA with Dunnett's multiple comparison tests to the NT condition [B] or the "R848 without ASO" condition [C] are shown).
- (D) Selected HPRT ASO sequences with low TLR7 inhibition. The conserved region between all the sequences is highlighted in light grey. The 2'OMe flanking regions are highlighted in dark grey. CUU motifs are in bold light grey, while UUC motifs are underlined.
- (E, F) WT THP-1 were incubated overnight with 100 nM ASOs. The next day, the cells were treated with lipofectamine 2000 (at 2.5  $\mu$ l/ml, to enhance cytoplasmic delivery of the ASOs), just before R848 stimulation (1  $\mu$ g/ml - for F only). Supernatants were collected after 8 h for IP-10 ELISA (F), and cells lysed for RNA purification after 24 h (E). (E) HPRT levels were reported to 18S, and normalised to NT condition. Data are averaged from four (E) or three (F) independent experiments in duplicate ( $\pm$  s.e.m and ordinary one-way ANOVA with Dunnett's multiple comparison tests to the "R848 without ASO" condition [F] or Mann-Whitney U tests [E] are shown). \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , \*\*\*\*  $P \leq 0.0001$ , ns: non-significant.

**Figure 6.** Preliminary screen of 48 ASOs targeting the human HPRT gene in HeLa cells. HeLa cells were reverse-transfected with indicated ASO quantities as detailed in Example 1, and HPRT levels measured by RT-qPCR after 24h incubation. HPRT levels were reported to SFRS9 expression, and further normalised to the average of the non-targeting ASONC1 and ASONC5 control conditions. Data shown represent the average of biological triplicates from one experiment ( $\pm$  s.e.m.).

**Figure 7.** ASO852-dT potentiation of TLR7 and TLR8 ligands. HEK-TLR8 cells expressing an NF- $\kappa$ B-luciferase reporter were treated with 100 nM ASO852-dT (or NT) for 20 min prior to stimulation with Loxoribine (5mM), CL075 (1 $\mu$ g/ml), Gardiquimod (1 $\mu$ g/ml). NF- $\kappa$ B-luciferase levels were measured after overnight incubation. Data are shown as fold increase to NT condition, and are averaged from two independent experiment in biological triplicate ( $\pm$  s.e.m. and ordinary one-way ANOVA with Mann-Whitney U tests are shown).

**Figure 8.** Basal activities of ASOs on TLR7, 8, 9 HEK cells and THP-1 cells. (A, B, C) HEK-TLR7 (A), TLR8 (B), and TLR9 (C) cells expressing an NF- $\kappa$ B-luciferase reporter were treated with 500 nM indicated ASOs. NF- $\kappa$ B-luciferase levels were measured after overnight incubation. (A, B) cells were treated with 1  $\mu$ g/ml R848 as positive control, alone or in the presence of ASO2 at 500 nM (for panel B only). (C) HEK-TLR9 cells were stimulated with 200 nM of ODN 2006 as a positive control. Fold increases relative to the NT condition are averaged from two (A, B) or three independent experiments (C) in biological triplicate ( $\pm$  s.e.m and ordinary one-way ANOVA with Dunnett's multiple comparison tests [A, B] or Tukey's multiple comparison tests to the NT condition and selected pairs of conditions [C] are shown). (D) WT THP-1 were pre-treated overnight with 100 nM ASO (except condition "852-dT 500nM" with 500 nM ASO used), and stimulated with 1  $\mu$ g/ml R848 for 8 h and IP-10 levels in supernatants determined by ELISA. Data shown are averaged from two independent experiments in biological triplicate ( $\pm$  s.e.m and ordinary one-way ANOVA with Dunnett's multiple comparison tests to the NT condition are shown). (A-D) Unless otherwise mentioned, differences to NT condition were non-significant. \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , \*\*\*\*  $P \leq 0.0001$ .

**Figure 9.** Motif-specific TLR8 potentiation. THP-1 cells and HEK-TLR8 cells expressing an NF- $\kappa$ B-luciferase reporter were pre-treated (overnight for THP-1, and ~30 min for HEK) with indicated concentrations of ASOs, prior to R848 stimulation for 7h (THP-1, IP-10 ELISA) or overnight for NF- $\kappa$ B-Luciferase. Data shown are averaged

from 3 independent experiments in biological triplicate. For HEK-TLR8, the NF- $\kappa$ B-luciferase values are reported to the R848 condition. All ASO conditions are with R848 co-stimulation. SEM and One-way ANOVA with Dunnett's multiple comparisons to R848 only condition are shown.

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**Figure 10.** Sequence-specific potentiation of uridine sensing by ASOs. THP-1 cells and HEK-TLR8 cells expressing an NF- $\kappa$ B-luciferase reporter or a RANTES-luciferase reporter were pre-treated (overnight for THP-1, and ~30 min for HEK) with 100 nM (THP-1) or 500 nM ASOs (HEKs), prior to uridine stimulation (20 mM) for 7h (THP-1, IP-10 ELISA) or overnight for HEK-TLR8 cells. Data shown are averaged from 3 (THP-1 and RANTES-Luc HEKs) or 2 (NF- $\kappa$ B-Luc HEKs) independent experiments in biological triplicate. For HEK-TLR8, the NF- $\kappa$ B-luciferase values are reported to R848 condition; for RANTES-luciferase values are reported to NT condition. All ASO conditions are with Uridine co-stimulation (except R848 conditions at 1  $\mu$ g/ml). SEM and One-way ANOVA with Dunnett's multiple comparisons to "Uridine only" condition are shown.

**Figure 11.** Identification of LNA and 2'MOE gapmer ASOs potentiating TLR8 sensing. HEK-TLR8 cells expressing an NF- $\kappa$ B-luciferase reporter were pre-treated for ~30 min with indicated concentrations of ASOs, prior to R848 stimulation (1  $\mu$ g/ml) overnight. Fold increases relative to the condition 'R848 without ASO' are averaged from biological duplicates (averaged data are provided in Table 1). Stimulations with 100 nM and 500 nM ASO were performed in independent experiments (data shown for each concentration is from a single experiment).

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**Figure 12.** Validation of LNA ASOs potentiating TLR8. THP-1 cells and HEK-TLR8 cells expressing an NF- $\kappa$ B-luciferase reporter were pre-treated (overnight for THP-1, and ~30 min for HEK) with indicated concentrations of LNA ASOs, prior to R848 stimulation for 7h (THP-1, IP-10 ELISA) or overnight for NF- $\kappa$ B-Luciferase. Data shown are averaged from 3 independent experiments in biological triplicate (THP-1) or duplicate (HEKs). For HEK-TLR8 cells, the NF- $\kappa$ B-luciferase values are reported to "R848 only" condition. All ASO conditions are with R848 co-stimulation. SEM and One-way ANOVA with Dunnett's multiple comparisons to R848 only condition are shown.

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**Figure 13.** Validation of 2'MOE ASOs potentiating TLR8. HEK-TLR8 cells expressing an NF- $\kappa$ B-luciferase reporter were pre-treated for ~30 min with 500 nM indicated 2'MOE ASOs, prior to R848 stimulation (1  $\mu$ g/ml) overnight. All ASO conditions are with R848 co-stimulation. Fold increases relative to the condition 'R848 without ASO' are averaged from biological replicate - data shown are from a single experiment ( $\pm$ SEM).

**Figure 14.** Potentiation of TLR8 sensing by dT20 does not persist after wash off of the oligonucleotides. Left: HEK-TLR8 cells expressing an NF- $\kappa$ B-luciferase reporter were pre-treated overnight with 500 nM dT20, prior to be washed (or not) and stimulated with R848 stimulation (1  $\mu$ g/ml) overnight. Data shown are averaged from 2 independent experiments in biological triplicate, and reported to R848 only condition. SEM and One-way ANOVA with Dunnett's multiple comparisons to R848 only condition are shown. Right: THP-1 cells were incubated with 100 nM indicated ASOs overnight (purple) or for 2.5 h, prior to R848 stimulation (1  $\mu$ g/ml) for ~7 h. IP-10 levels were measured by ELISA. Data shown are from a single experiment in biological triplicate (each dot represents a biological replicate). All ASO conditions are with R848 co-stimulation.

**Figure 15.** Co-culture of phagocytes with ASO-transfected cells leads to sequence specific TLR8 potentiation. HEK WT cells were transfected with 500 nM ASO3 (non-TLR8 potentiating) or 500 nM 852dT (strongly potentiating TLR8), for 4 h, prior to UV treatment (254 nm at 120 mJ/cm<sup>2</sup>), extensive washing (2x 5ml - to remove untransfected ASOs), and co-culture with 6-day PMA-differentiated THP-1 overnight, before 24 h R848 stimulation (at 5  $\mu$ g/ml). TNF $\alpha$  levels were measured by ELISA. Data shown are relative to ASO3 or ASO 852dT conditions, and averaged from 2 independent experiments with biological replicate. SEM and unpaired two-tailed t-test are shown.

**Figure 16.** TLR8-potentiation by fully a 2'Ome modified ASO. HEK-TLR8 cells expressing an NF- $\kappa$ B-luciferase reporter were pre-treated for ~30 min with 500 nM indicated 2'MOE ASOs, prior to R848 stimulation (1  $\mu$ g/ml) overnight. All ASO conditions are with R848 co-stimulation. Fold increases relative to the condition 'R848 without ASO' are averaged from 3 independent experiments in biological triplicate ( $\pm$ SEM and One-way ANOVA with Dunnett's multiple comparisons to R848 only condition are shown).

**Figure 17.** 5'-end CUU motifs modulate TLR7 sensing in the context of 2'OMe ASOs, not LNA ASOs. HEK-TLR7 cells expressing an NF- $\kappa$ B-luciferase reporter were pre-treated for ~30 min with 500 nM indicated 2'MOE ASOs, prior to R848 stimulation (1  $\mu$ g/ml) overnight. All ASO conditions are with R848 co-stimulation. Data shown are averaged from 3 independent experiments in biological triplicate, and reported to R848 only condition. SEM and One-way ANOVA with Dunnett's multiple comparisons to "660-Mut" condition are shown.

**Figure 18.** Identification of LNA and 2'MOE gapmer ASOs that do not inhibit TLR7 sensing. HEK-TLR7 cells expressing an NF- $\kappa$ B-luciferase reporter were pre-treated for ~30 min with indicated concentrations of ASOs, prior to R848 stimulation (1  $\mu$ g/ml) overnight. Fold increases relative to the condition 'R848 without ASO' are averaged from biological duplicates (averaged data are provided in Table 2). Stimulations with 100 nM and 500 nM ASO were performed in independent experiments (data shown for each concentration is from a single experiment).

**Figure 19.** Validation of LNA and 2'MOE gapmer ASOs with limited TLR7 inhibition. HEK-TLR7 cells expressing an NF- $\kappa$ B-luciferase reporter were pre-treated for ~30 min with 500 nM indicated 2'MOE ASOs, prior to R848 stimulation (1  $\mu$ g/ml) overnight. All ASO conditions are with R848 co-stimulation. Data shown are averaged from 3 independent experiments (for LNA) or a single experiment (for 2'MOE) in biological triplicate, and reported to R848 only condition. SEM and One-way ANOVA with Dunnett's multiple comparisons to "R848 only" condition are shown for LNA ASOs (left). Hatched bars refer to ASOs with a 5' +C+C motif. For the 2'MOE experiment, the inventors note that E1 and a few other ASOs (not shown) entirely ablated TLR7 sensing – but G1-A2-C1-A9 did not.

#### **BRIEF DESCRIPTION ON THE SEQUENCES**

SEQ ID NO: 1 and SEQ ID NO: 2 represent the nucleotide sequences of negative targeting controls ASOs from Table 1. SEQ ID NO:3 through SEQ ID NO:20 represent the nucleotide sequences of ASOs targeting human cGAS mRNA and modified versions thereof from Table 1. SEQ ID NO: 21 and SEQ ID NO: 22 represent the nucleotide sequences of ASO852 and ASO852-DT from Table 1. SEQ ID NO: 23 and SEQ ID NO:24 represent the nucleotide sequences of ASO2504 and ASO2504-dT from Table 1. SEQ ID NO: 25 represents the nucleotide sequences of dT20 from Table 1. SEQ ID NO:26 through SEQ ID NO:34 represent the nucleotide sequences of Hs HPRT F517,

Hs HPRT R591, Hs HPRT P554 FAM, Hs SFRS9 F594, Hs SFRS9 R690, Hs SFRS9 P625 HEX, ODN 2006, ISD70-FWD and ISD70-REV respectively from Table 1.

SEQ ID NO:35 through SEQ ID NO:82 represent the nucleotide sequences of CDKN2B-AS1 ASOs from Table 2. SEQ ID NO:83 through SEQ ID NO:130 represent the nucleotide sequences of CTNNB1 ASOs from Table 2. SEQ ID NO:131 through SEQ ID NO:178 represent the nucleotide sequences of EGFR ASOs from Table 2. SEQ ID NO:179 through SEQ ID NO:226 represent the nucleotide sequences of LINC-PINT ASOs from Table 2.

SEQ ID NO:227 through SEQ ID NO:273 represent the nucleotide sequences of HPRT ASOs from Table 3. SEQ ID NO:274 through SEQ ID NO:282 represent the nucleotide sequences of ASO1-UC, ASO2 LNA, ASO2-LNA Mut1, ASO2-LNA Mut2, ASO 660, ASO 660-Mut, C2Mut-1, C2Mut1-PS, C2Mut1-2OMe respectively of Table 4. SEQ ID NO:283 through SEQ ID NO:373 represent the LNA-modified nucleotide sequences of Table 5. SEQ ID NO:374 through SEQ ID NO:449 represent the 2'-MOE-modified nucleotide sequences of Table 6.

## **DETAILED DESCRIPTION OF THE INVENTION**

### **General Techniques and Definitions**

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in oligonucleotide design, molecular genetics, antisense oligonucleotides, gene silencing, gene expression and biochemistry).

Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratory (1988), and J.E. Coligan et al. (editors) Current Protocols in Immunology, John Wiley & Sons (including all updates until present).

The term “and/or”, e.g., “X and/or Y” shall be understood to mean either “X and Y” or “X or Y” and shall be taken to provide explicit support for both meanings or for either meaning.

As used herein, the term about, unless stated to the contrary, refers to +/- 10%,  
5 more preferably +/-5%, more preferably +/-1%, of the designated value.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

10 By “consisting essentially of” in the context of an oligonucleotide sequence is meant the recited oligonucleotide sequence together with an additional one, two or three nucleic acids at the 5' or 3' end thereof.

As used herein, the phrase “does not inhibit Toll-like receptor 7 (TLR7) activity” or variations thereof means that after administration to an animal of an oligonucleotide  
15 of the invention the animal is still able to elicit a TLR7 based immune response, such as to a pathogen. In an embodiment, the TLR7 based immune response in the presence of the oligonucleotide is at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 97%, 99% or 100% of the response in the absence of the oligonucleotide.

Similarly, the phrase “reducing the Toll-like receptor 7 (TLR7) inhibitory activity  
20 of an oligonucleotide” or the like means that after being modified in accordance with the invention, an animal administered with the modified oligonucleotide is able to mount a stronger TLR7 based immune response when compared to the starting (unmodified) oligonucleotide.

As used herein, the phrase “potentiates Toll-like receptor 8 (TLR8) activity” and  
25 the like means that after administration to an animal of an oligonucleotide of the invention the animal has an enhanced (increased) TLR8 based immune response.

As used herein, an “immune response modifier” refers to any agent that mimics, augments, or require participation of host immune cells for optimal effectiveness, and/or has a known ability to activate, augment, or enhance specific immune responses.  
30 Examples of immune response modifiers include, but are not limited to, Toll-like receptor (TLR) agonists including Resiquimod (R848), Loxoribine, Isatoribine, Imiquimod, CL075, CL097, CL264, CL307, 852A, and/or TL8-506. Other Toll-like receptor (TLR) agonists can include a base analogue (including: a guanosine analogue, a deaza-adenosine analogue, an imidazoquinoline or a derivative, a hydroxyadenine  
35 compound or a derivative, a thiazoloquinolone compound or a derivative, a benzazepine compound or a derivative) and/or an RNA molecule.

The phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms that are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, and/or other problem or  
5 complication, commensurate with a reasonable benefit/risk ratio.

As used herein, the terms “treating”, “treat” or “treatment” include administering a therapeutically effective amount of a compound(s) described herein sufficient to reduce or eliminate at least one symptom of a disease.

As used herein, the terms “preventing”, “prevent” or “prevention” include  
10 administering a therapeutically effective amount of a compound(s) described herein sufficient to stop or hinder the development of at least one symptom of a disease.

#### Oligonucleotides

In the context of this invention, the term "oligonucleotide" refers to an oligomer  
15 or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), wherein the polymer or oligomer of nucleotide monomers contains any combination of nucleobases (referred to in the art and herein as simply as "base"), modified nucleobases, sugars, modified sugars, phosphate bridges, or modified phosphorus atom bridges (also referred to herein as "internucleotidic linkage").

20 Oligonucleotides can be single-stranded or double-stranded or a combination thereof. A single-stranded oligonucleotide can have double-stranded regions and a double-stranded oligonucleotide can have single-stranded regions (such as a microRNA or shRNA).

"Gapmer" refers to an oligonucleotide comprising an internal region having a  
25 plurality of nucleosides that support RNase H cleavage positioned between external regions having one or more nucleosides, wherein the nucleosides comprising the internal region are chemically distinct from the nucleoside or nucleosides comprising the external regions. The internal region may be referred to as the "gap" and the external regions may be referred to as the "wings."

30 As used herein, a “target” such as a “target gene” or “target polynucleotide” refers to a molecule upon which an oligonucleotide of the invention directly or indirectly exerts its effects. Typically, the oligonucleotide of the invention or portion thereof and the target, or a product of the target such as mRNA encoded by a gene, or portion thereof, are able to hybridize under physiological conditions.

35 As used herein, the phrase “reduces expression of the target gene” or the like refers to an oligonucleotide of the invention reducing the ability of a gene to exert its

biological effect. This can be directly or indirectly achieved by reduction in the amount of RNA encoded by the gene and/or reduction of the amount of protein translated from an RNA.

Typically, an oligonucleotide of the invention will be synthesized *in vitro*.

5 However, in some instances where modified bases and backbone are not required they can be expressed *in vitro* or *in vivo* in a suitable system such as by a recombinant virus or cell.

10 An oligonucleotide of the invention may be conjugated to one or more moieties or groups which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. These moieties or groups may be covalently bound to functional groups such as primary or secondary hydroxyl groups. Exemplary moieties or groups include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate  
15 groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins and dyes.

In particular embodiments, the oligonucleotide described herein may comprise a synthetic oligonucleotide sequence. As used herein, a "synthetic oligonucleotide sequence" refers to an oligonucleotide sequence which lacks a corresponding sequence  
20 that occurs naturally. By way of example, a synthetic oligonucleotide sequence is not complementary to a specific RNA molecule, such as one encoding an endogenous polypeptide. As such, the synthetic oligonucleotide sequence is suitably not capable of interfering with a post-transcriptional event, such as RNA translation.

As used herein, an oligonucleotide "variant" shares a definable nucleotide  
25 sequence relationship with a reference nucleic acid sequence. The reference nucleic acid sequence may be one of those provided in Tables 1 through 6 (e.g., SEQ ID NOs. 1-449), for example. The "variant" oligonucleotide may have one or a plurality of nucleic acids of the reference nucleic acid sequence deleted or substituted by different nucleic acids. Preferably, oligonucleotide variants share at least 70% or 75%, preferably at least 80%  
30 or 85% or more preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a reference nucleic acid sequence.

#### *Modified Bases*

Oligonucleotides of the invention may have nucleobase ("base") modifications or  
35 substitutions.

Examples include oligonucleotides comprising one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C1 to C10 alkyl or C2 to C10 alkenyl and alkynyl. In one embodiment, the oligonucleotide  
 5 comprises one of the following at the 2' position:  $O[(CH_2)_nO]_mCH_3$ ,  $O(CH_2)_nOCH_3$ ,  $O(CH_2)_nNH_2$ ,  $O(CH_2)_nCH_3$ ,  $O(CH_2)_nONH_2$ , and  $O(CH_2)_nON[(CH_2)_nCH_3]_2$ , where n and m are from 1 to about 10.

Further examples include of modified oligonucleotides include oligonucleotides comprising one of the following at the 2' position: C1 to C10 lower alkyl, substituted  
 10 lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the  
 15 pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties.

In one embodiment, the modification includes 2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub> (also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., 1995), that is, an alkoxyalkoxy group. In some embodiments, the modification does not  
 20 comprise 2'-MOE. In a further embodiment, the modification includes 2'-dimethylaminoethoxyethoxy, that is, a  $O(CH_2)_2ON(CH_3)_2$  group (also known as 2'-DMAOE), or 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylamino-ethoxy-ethyl or 2'-DMAEOE), that is, 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>3</sub>)<sub>2</sub>.

Other modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), 2'-allyl (2'-CH<sub>2</sub>-CH=CH<sub>2</sub>), 2'-O-allyl (2'-O-CH<sub>2</sub>-CH=CH<sub>2</sub>) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. In one embodiment a 2'-arabino modification is 2'-F.  
 25

Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked  
 30 oligonucleotides and the 5' position of the 5' terminal nucleotide.

Oligonucleotides may also have sugar mimetics, such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, US 4,981,957, US 5,118,800, US  
 35 5,319,080, US 5,359,044, US 5,393,878, US 5,446,137, US 5,466,786, US 5,514,785, US 5,519,134, US 5,567,811, US 5,576,427, US 5,591,722, US 5,597,909, US

5,610,300, US 5,627,053, US 5,639,873, US 5,646,265, US 5,658,873, US 5,670,633, US 5,792,747, and US 5,700,920.

A further modification of the sugar includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring, thereby forming a bicyclic sugar moiety. In one embodiment, the linkage is a methylene (-CH<sub>2</sub>-) group bridging the 2' oxygen atom and the 4' carbon atom, wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226. In some embodiments, however, the modification does not comprise LNA.

Modified nucleobases include other synthetic and natural nucleobases such as, for example, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-CC-CH<sub>3</sub>) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Further modified nucleobases include tricyclic pyrimidines, such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as, for example, a substituted phenoxazine cytidine (e.g., 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one).

Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example, 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in US 3,687,808, those disclosed in J.I. Kroschwitz (editor), The Concise Encyclopedia of Polymer Science and Engineering, pages 858-859, John Wiley and Sons (1990), those disclosed by Englisch et al. (1991), and those disclosed by Y.S. Sanghvi, Chapter 15: Antisense Research and Applications, pages 289-302, S.T. Crooke, B. Lebleu (editors), CRC Press, 1993.

Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligonucleotide. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-

aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 oC. In one embodiment, these nucleobase substitutions are combined with 2'-O-methoxyethyl sugar modifications.

5 Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, US 3,687,808, US 4,845,205, US 5,130,302, US 5,134,066, US 5,175,273, US 5,367,066, US 5,432,272, US 5,457,187, US 5,459,255, US 5,484,908, US 5,502,177, US 5,525,711, US 5,552,540, US 5,587,469, US 5,594,121, US 5,596,091,  
10 US 5,614,617, US 5,645,985, US 5,830,653, US 5,763,588, US 6,005,096, US 5,681,941 and US 5,750,692.

Unless stated to the contrary, reference to an A, T, G, U or C can either mean a naturally occurring base or a modified version thereof.

In particular embodiments, two or more bases (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,  
15 12, 13, 14, 15, 16, 17, 18, 19, 20 bases inclusive of any range therein) of the oligonucleotide described herein are modified. In some embodiments, all bases of the oligonucleotide described herein are modified. In alternative embodiments, no bases of the oligonucleotide described herein are modified.

## 20 *Backbones*

Oligonucleotides of the present disclosure include those having modified backbones or non-natural internucleoside linkages. Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone.

25 Modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and  
30 aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide  
35 linkage, that is, a single inverted nucleoside residue which may be abasic (the nucleobase

is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, US 3,687,808, US 4,469,863, US 4,476,301, US 5,023,243, US 5,177,196, US 5,188,897, US 5,264,423, US 5,276,019, US 5,278,302, US 5,286,717, US 5,321,131, US 5,399,676, US 5,405,939, US 5,453,496, US 5,455,233, US 5,466,677, US 5,476,925, US 5,519,126, US 5,536,821, US 5,541,306, US 5,550,111, US 5,563,253, US 5,571,799, US 5,587,361, US 5,194,599, US 5,565,555, US 5,527,899, US 5,721,218, US 5,672,697 and US 5,625,050.

Modified oligonucleotide backbones that do not include a phosphorus atom therein include, for example, backbones formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts.

Representative United States patents that teach the preparation of the above oligonucleotides include, but are not limited to, US 5,034,506, US 5,166,315, US 5,185,444, US 5,214,134, US 5,216,141, US 5,235,033, US 5,264,562, US 5,264,564, US 5,405,938, US 5,434,257, US 5,466,677, US 5,470,967, US 5,489,677, US 5,541,307, US 5,561,225, US 5,596,086, US 5,602,240, US 5,610,289, US 5,602,240, US 5,608,046, US 5,610,289, US 5,618,704, US 5,623,070, US 5,663,312, US 5,633,360, US 5,677,437, US 5,792,608, US 5,646,269 and US 5,677,439.

### 30 *Antisense Oligonucleotides*

The term "antisense oligonucleotide" shall be taken to mean an oligonucleotide that is complementary to at least a portion of a specific mRNA molecule, such as encoding an endogenous polypeptide and capable of interfering with a post-transcriptional event such as mRNA translation. The use of antisense methods is well known in the art (see for example, G. Hartmann and S. Endres, *Manual of Antisense Methodology*, Kluwer (1999)).

In one embodiment, the antisense oligonucleotide hybridises under physiological conditions, that is, the antisense oligonucleotide (which is fully or partially single stranded) is at least capable of forming a double stranded polynucleotide with mRNA, such as encoding an endogenous polypeptide, under normal conditions in a cell.

5        Antisense oligonucleotides may include sequences that correspond to the structural genes or for sequences that effect control over the gene expression or splicing event. For example, the antisense sequence may correspond to the targeted coding region of endogenous gene, or the 5'-untranslated region (UTR) or the 3'-UTR or combination of these. It may be complementary in part to intron sequences, which may be spliced out  
10        during or after transcription, preferably only to exon sequences of the target gene. In view of the generally greater divergence of the UTRs, targeting these regions provides greater specificity of gene inhibition.

      The antisense oligonucleotide may be complementary to the entire gene transcript, or part thereof. The degree of identity of the antisense sequence to the targeted  
15        transcript should be at least 90% and more preferably 95-100%. The antisense RNA or DNA molecule may of course comprise unrelated sequences which may function to stabilize the molecule such as described herein.

#### *Gene Silencing*

20        Oligonucleotide molecules, particularly RNA, may be employed to regulate gene expression. The terms "RNA interference", "RNAi" or "gene silencing" refer generally to a process in which a dsRNA molecule reduces the expression of a nucleic acid sequence with which the double-stranded RNA molecule shares substantial or total homology. However, it has been shown that RNA interference can be achieved using  
25        non-RNA double stranded molecules (see, for example, US 20070004667).

      The double-stranded regions should be at least 19 contiguous nucleotides, for example about 19 to 23 nucleotides, or may be longer, for example 30 or 50 nucleotides, or 100 nucleotides or more. The full-length sequence corresponding to the entire gene transcript may be used. Preferably, they are about 19 to about 23 nucleotides in length.

30        The degree of identity of a double-stranded region of a nucleic acid molecule to the targeted transcript should be at least 90% and more preferably 95-100%. The nucleic acid molecule may of course comprise unrelated sequences which may function to stabilize the molecule.

      The term "short interfering RNA" or "siRNA" as used herein refers to a  
35        polynucleotide which comprises ribonucleotides capable of inhibiting or down regulating gene expression, for example by mediating RNAi in a sequence-specific manner,

wherein the double stranded portion is less than 50 nucleotides in length, preferably about 19 to about 23 nucleotides in length. For example the siRNA can be a nucleic acid molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide  
5 sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siRNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary. The two strands can be of different length.

10 As used herein, the term siRNA is meant to be equivalent to other terms used to describe polynucleotides that are capable of mediating sequence specific RNAi, for example micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid (siNA), short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA  
15 (ptgsRNA), and others. In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, translational inhibition, or epigenetics. For example, siRNA molecules can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level. In a non-limiting example,  
20 epigenetic regulation of gene expression by siRNA molecules can result from siRNA mediated modification of chromatin structure to alter gene expression.

By "shRNA" or "short-hairpin RNA" is meant an RNA molecule where less than about 50 nucleotides, preferably about 19 to about 23 nucleotides, is base paired with a complementary sequence located on the same RNA molecule, and where said sequence  
25 and complementary sequence are separated by an unpaired region of at least about 4 to about 15 nucleotides which forms a single-stranded loop above the stem structure created by the two regions of base complementarity. An Example of a sequence of a single-stranded loop includes: 5' UUCAAGAGA 3'.

Included shRNAs are dual or bi-finger and multi-finger hairpin dsRNAs, in which  
30 the RNA molecule comprises two or more of such stem-loop structures separated by single-stranded spacer regions.

#### Design and Testing of Candidate Oligonucleotides

As the skilled person is aware, in addition to design elements of the invention,  
35 there are many known factors to be considered when producing an oligonucleotide. The specifics depend on the purpose of the oligonucleotide but include features such as

strength and stability of the oligonucleotide-target nucleic acid interaction, such as the mRNA secondary structure, thermodynamic stability, the position of the hybridization site, and/or functional motifs.

Some methods the invention involve scanning a target polynucleotide, or  
5 complement thereof, for specific features. This can be done by eye or using computer programs known in the art. Software programs which can be used to design, analyse and predict functional properties of antisense oligonucleotides include Mfold, Sfold, NUPACK, Nanofolder, Hyperfold, and/or RNA designer. Software programs which can be used to design, analyse and predict functional properties of oligonucleotides for gene  
10 silencing include dsCheck, E-RNAi and/or siRNA-Finder.

In one embodiment, available software is used to select potentially useful oligonucleotides, and then these are scanned for desired features as described herein. Alternatively, software could readily be developed to scan a target polynucleotide, or complement thereof, for desired features as described herein.

15 Once synthesized, candidate oligonucleotides can be tested for their desired activity using standard procedures in the art. This may involve administering the candidate to cells *in vitro* expressing the gene of interest and analysing the amount of gene product such as RNA and/or protein. In another example, the candidate is administered to an animal, and the animal screened for the amount of target RNA and/or  
20 protein and/or using a functional assay. In another embodiment, the oligonucleotide is tested for its ability to hybridize to a target polynucleotide (such as mRNA).

In some examples expression and oligonucleotide activity can be determined by mRNA reverse transcription quantitative real-time PCR (RT-qPCR). For example, RNA can be extracted and purified from cells which have been incubated with a candidate  
25 oligonucleotide. cDNA is then synthesized from isolated RNA and RT-qPCR can be performed, using methods and reagents known the art. In one example, RNA can be purified from cells using the ISOLATE II RNA Mini Kit (Bioline) and cDNA can be synthesized from isolated RNA using the High-Capacity cDNA Archive kit (Thermo Fisher Scientific) according to the manufacturer's instructions. RT-qPCR can be  
30 performed using the Power SYBR Green Master Mix (Thermo Fisher Scientific) on the HT7900 and QuantStudio 6 RT-PCR system (Thermo Fisher Scientific), according to manufacturer's instructions.

#### *Testing for inhibition of TLR7 activity*

35 Some aspects of the present invention involve testing for inhibition of TLR7 activity which can be determined using any method known in the art. In some

embodiments, TLR7 activity in cells may be measured by expression and/or secretion of one or more proinflammatory cytokines (e.g. TNF $\alpha$ , IP-10), and/or activation or expression of transcription factors (e.g. NF- $\kappa$ B).

The ability of an oligonucleotide to inhibit TLR7 activity can, for example, be analysed by incubating cells which express TLR7 with an oligonucleotide, then stimulating said cells with a TLR7 agonist, and analysing the overall TLR7 response in the cell population, or analysing the proportion of cells having TLR7-positive activity after a defined period of time.

In such examples, inhibition of TLR7 activity can be identified by observation of an overall decreased TLR7 response of the cell population, or a lower proportion of cells having TLR7-positive activity as compared to positive control condition in which cells are treated with a TLR7 agonist in the absence of the oligonucleotide (or in the presence of an appropriate control inhibitory agent). In one example, 293XLhTLR7 (referred to as HEK-TLR7) cells are transfected with pNF- $\kappa$ B-Luc4 reporter, incubated with an oligonucleotide, and then stimulated with R848. TLR7 activity can be determined by a luciferase assay, which measures activated NF- $\kappa$ B by luminescence. TLR7 activity can also be analysed by measuring cytokine levels, for example by ELISA.

#### *Testing for potentiating TLR8 activity*

Some aspects of the present invention involve testing for potentiation of TLR8 activity which can be determined using any method known in the art. In some embodiments TLR8 activity in cells may be measured by expression and/or secretion of one or more proinflammatory cytokines (e.g. TNF $\alpha$ , IP-10), and/or activation or expression of transcription factors (e.g. NF- $\kappa$ B).

The ability of an oligonucleotide to potentiate TLR8 activity can, for example, be analysed by incubating cells which express TLR8 with an oligonucleotide, then stimulating said cells with a TLR8 agonist, and analysing the overall TLR8 response in the cell population, or analysing the proportion of cells having TLR8-positive activity after a defined period of time.

In such examples, potentiation of TLR8 activity can be identified by observation of an overall decreased TLR8 response of the cell population, or a higher proportion of cells having TLR8-positive activity as compared to a negative control condition in which cells are treated with TLR8 agonist in the absence of the oligonucleotide (or in the presence of an appropriate control non-potentiating agent). In one example, 293XLhTLR8 (referred to as HEK-TLR8) cells are transfected with pNF- $\kappa$ B-Luc4 reporter, incubated with an oligonucleotide, and then stimulated with R848. TLR8

activity can be determined by a luciferase assay, which measures activated NF- $\kappa$ B by luminescence. TLR8 activity can also be analysed by measuring cytokine levels, for example by ELISA.

‘Potentiation’ refers to an increase in a functional property relative to a control condition. Potentiation of TLR8 activity may be greater than about 100%, *e.g.* about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold, about 10 fold, about 11 fold, about 12 fold, about 13 fold, about 14 fold, about 15 fold, about 20 fold or about 50 fold. Preferably, the level of TLR8 potentiation is between about 2 fold and 50 fold, between about 2 fold and 20 fold, and/or between about 5 fold and 20 fold greater.

### Uses

Oligonucleotides of the invention are designed to be administered to an animal. In one example, the animal is a vertebrate. For example, the animal can be a mammal, avian, chordate, amphibian or reptile. Exemplary subjects include but are not limited to human, primate, livestock (*e.g.* sheep, cow, chicken, horse, donkey, pig), companion animals (*e.g.* dogs, cats), laboratory test animals (*e.g.* mice, rabbits, rats, guinea pigs, hamsters), captive wild animal (*e.g.* fox, deer). In one example, the mammal is a human.

Oligonucleotides of the invention can be used to target any gene/polynucleotide/function of interest. Typically, the oligonucleotide is used to modify a trait of an animal, more typically to treat or prevent a disease. In a preferred embodiment, the disease will benefit from the animal being able to mount a TLR7 and/or TLR8 response following administration of the oligonucleotide, in particular where the TLR7 response is not inhibited and/or the TLR8 response is potentiated.

Diseases which can be treated or prevented using an oligonucleotide of the invention include, but are not limited, to cancer (for example breast cancer, ovarian cancer, cancers of the central nervous system, gastrointestinal cancer, bladder cancer, skin cancer, lung cancer, head and neck cancers, haematological and lymphoid cancers, bone cancer) rare genetic diseases, neuromuscular and neurological diseases (for example, spinal muscular atrophy, Amyotrophic Lateral Sclerosis, Duchenne muscular dystrophy, Huntington’s disease, Batten disease, Parkinson’s disease, amyotrophic lateral sclerosis, Ataxia-telangiectasia, cerebral palsy) viruses (for example, cytomegalovirus, hepatitis C virus, Ebola hemorrhagic fever virus, human immunodeficiency virus, coronaviruses), cardiovascular disease (for example, familial hypercholesterolemia, hypertriglyceridemia), autoimmune and inflammatory diseases

(for example arthritis, lupus, pouchitis, psoriasis, asthma), and non-alcoholic and alcoholic fatty liver diseases.

- Examples of target genes (polynucleotides) of oligonucleotides of the invention include, but not limited to, PLK1ERBB2, PIK3CA, ERBB3, HDAC1, MET, EGFR,
- 5 TYMS, TUBB4B, FGFR2, ESR1, FASN, CDK4, CDK6, NDUFB4, PPAT, NDUFB7, DNMT1, BCL2, ATP1A1, HDAC3, FGFR1, NDUFS2, HDAC2, NDUFS3, HMGCR, IGF1R, AKT1, BCL2L1, CDK2, MTOR, PDPK1, CSNK2A1, PIK3CB, CDK12, MCL1, ATR, PLK4, MEN1, PTK2, FZD5, KRAS, WRN, CREBBP, NRAS, MAT2A, RHOA, TPX2, PPP2CA, ALDOA, RAE1, SKP1, ATP5A1, EIF4G1, CTNNB1, TFRC,
  - 10 CDH1, CCNE1, CLTC, METAP2, GRB2, MDM4, SLC16A1, FERMT2, ENO1, STX4, SF3B1, RBBP4, FEN1, MRPL28, CCNA2, PTPN11, SAE1, KMT2D, APC, CAD, NAMPT, OGT, HSPA8, USP5, CSNK1A1, PGD, VRK1, SEPSECS, SUPT4H1, DNAJC9, TRIAP1, DLD, PTPN7, VDAC1, STAT3, TCEB2, ADSL, GMPS, DHPS, METAP1, TAF13, CFL1, SCD, RBM39, PGAM1, FNTB, PPP2R1A, ARF1, UBE2T,
  - 15 UMPS, MYC, PRMT5, EIF4G2, SKP2, STAG2, ATF4, WDR77, ILK, METTL16, SOD1, DDX6, FURIN, AARS, FNTA, PABPC1, RANBP2, CDC25B, SLC2A1, CENPE, ADAR, CDC42, RNF31, CCNC, PRIM1, SLC38A2, SNUPN, PDCD6IP, RTN4IP1, VMP1, TGFB1, TXN, UBE2N, UAP1, RAC1, GGPS1, RAB10, RAB6A, TPI1, RPE, THG1L, UBE2D3, RHEB, PKM, GMNN, HGS, NCKAP1, NUP98,
  - 20 SMARCA2, RNF4, DDX39B, ACLY, XPO1, PPP1R8, YAP1, MTHFD1, LPAR1, TAF1, UROD, STXBP3, HSP90B1, VHL, EFR3A, FECH, MRPL44, AIFM1, MAGOH, MRPL17, SUZ12, RNMT, RAB1B, PNPT1, RAD1, WDR48, PITRM1, MRPL47, AP2M1, EIF4A1, UBE2C, LONP1, VPS4A, SNRNP25, TUBGCP6, DNMT2, UBE2M, EXOSC9, TAF1B, CDC37, ATP6V1G1, POP1, JUP, PRPS1, GPX4, CFLAR,
  - 25 CHMP4B, ACTB, ACTR1A, PTPN23, SHC1, TRPM7, SLC4A7, HSPD1, XRN1, WDR1, ITGB5, UBR4, ATP5B, CPD, TUFM, MYH9, ATP5F1, ATP6V1C1, SOD2, PFAS, NFE2L2, ARF4, ITGAV, DHX36, KIF18A, DDX5, XRCC5, DNAJC11, ZBTB80S, NCL, SDHB, ATP5C1, NDC1, SNF8, CUL3, SLC7A1, ASNA1, EDF1, TMED10, CHMP6, ARIH1, DDOST, RPL28, DIMT1, CMPK1, PPIL1, PPA2, SMAD7,
  - 30 CEP55, MVD, MVK, PDS5A, KNTC1, CAPZB, GMPPB, TPT1, ACIN1, SAR1A, TAF6L, PTBP1, PAK2, CRKL, NHLRC2, INO80, SLC25A3, ACTR3, DDX3X, HUWE1, TBCA, IK, SSBP1, ARPC4, SLC7A5, OSGEP, PDCD2, TRAF2, SNAP23, RPN1, EIF5A, GEMIN4, BMPR1A, AHCYL1, CHMP5, TRAPPC1, LRP8, ARID2, UBE2L3, STAMBP, KDSR, UQCRC2, PNN, USP7, TBCD, ATP6V0E1, PCYT1A,
  - 35 TAZ, POLRMT, CELSR2, TERF1, BUB1, YRDC, SMG6, TBX3, SLC39A10, IPO13, CDIPT, UBA5, EMC7, FERMT1, VEZT, CCND1, CCND2, FPGS, JUN, PPM1D,

- PGGT1B, NPM1, GTF2A1, MBTPS1, HMGCS1, LRR1, HSD17B12, LCE2A, NUP153, FOSL1, IRS2, CYB5R4, PMPCB, ARHGEF7, TRRAP, NRBP1, ARMC7, MOCS3, TIPARP, SEC61A1, PFDN5, MYB, IRF4, STX5, MYCN, FOXA1, SOX10, GATA3, ZEB2, MYBL2, MFN2, TBCB, KLF4, TRIM37, CEBPA, STAG1, POU2AF1,
- 5 HYPK, FLI1, NCAPD2, MAF, NUP93, RBBP8, HJURP, SMARCB1, SOCS3, GRWD1, NKX2-1, FDXR, SPDEF, SBDS, SH3GL1, KLF5, CNOT3, ZNF407, CPSF1, RPTOR, EXT1, SMC1A, GUK1, TIMM23, FAU, ACO2, ALG1, CCNL1, SCAP, SRSF6, SPAG5, SOX9, LDB1, ASPM, LIG1, TFDP1, RPAIN, CENPA, MIS12, ILF3, HSCB, ERCC2, SOX2, ARFRP1, PMF1, POLR3E, MAD2L2, PELP1, NXT1, WDHD1,
- 10 ZWINT, E2F3, FZR1, JUNB, OGDH, NOB1, SKA3, TACC3, UTP14A, XRN2, SMG5, IDH3A, CIAO1, COQ4, ZFP36L1, CDCA5, PRKRA, PFDN6, PAK1IP1, PSTK, EDC4, UTP18, TOMM22, CASC5, PTTG1, RBBP5, PPP1R12A, FARS2, FOXM1, SIN3A, BUB1B, GNB1L, SMC5, SARS2, SYNCRIP, IPPK, FANCD2, WDR46, FANCI, DCP2, RFC2, RNF20, DMAP1, MED23, MBNL1, CTPS1, TBP, MMS19, RAD51C,
- 15 CDS2, NONO, USP18, PARS2, FBXW11, SUMO2, RRP12, FAM50A, URB2, MCM4, SLC25A28, IPO7, MAX, SFSWAP, SBNO1, DPAGT1, TINF2, BRCA2, NUP50, RPIA, EP400, IKBKAP, KIF14, RTTN, CCDC115, GEMIN6, WWTR1, BCS1L, GTF3A, SCYL1, NELFB, DDX39A, TRA2B, SYVN1, ISL1, CYB5B, ACSL3, DPH3, E2F1, IREB2, SREBF1, SMC6, IRF8, ID1, PDCD11, SNAPC2, TIMM17A, ANAPC10,
- 20 NUP85, SEH1L, VBP1, NUDC, MTX2, RPP25L, ISY1, LEMD2, ATP5D, EXOSC2, TAF1C, PPIL4, SEPHS2, HNRNPH1, CTR9, CDC26, TIMM13, FAM96B, CEBPZ, UFL1, ZNF236, COPG1, TPR, MIOS, UBE2G2, MED12, GTF3C1, PPP2R2A, UBIAD1, WTAP, MYBBP1A, NUP88, NELFCD, WDR73, RTCB, CEP192, GTF3C5, LENG1, RINT1, MED24, COX6B1, DCTN6, SLC25A38, LYRM4, STRAP, TTF2,
- 25 DDX27, GTF2F1, ZNHIT2, BCLAF1, WDR18, GTF2H2C, NDE1, TIMM9, CHMP7, IPO11, TGIF1, NOC4L, EXOSC6, WDR24, INTS6, DDX41, UBE2S, ARGLU1, SHOC2, ATP5J, CSTF2, RPP30, NHP2, GRHL2, RPL22L1, WDR74, UTP23, CCDC174, RPP21, UBE2J2, GEMIN8, ATP6V0B, KIAA1429, PNO1, MED22, ENY2, THOC7, DDX19A, SUGP1, PELO, ELAC2, CHCHD4, RNPC3, INTS3, PSMG4,
- 30 UQCRC1, TAF1A, TSR1, UTP6, TRMT5, EIF1AD, GTF3C2, DCTN3, GPS1, WDR7, EXOSC8, KANSL1, SPRTN, KANSL3, EXOSC5, PRCC, TRNAU1AP, EIF3J, TAMM41, HAUS6, OIP5, HAUS5, TAF6, MRPS22, MRPS34, WBP11, COG8, DHX38, DNLZ, LAGE3, FUBP1, MED26, SLC7A6OS, MARS2, RBM28, ASCC3, PSMG3, TUBGCP5, PCF11, or LASIL.

In an embodiment, the gene to be targeted includes PKN3, VEGFA, KIF11, MYC, EPHA2, KRAS (G12), ERBB3, BIRC5, HIF1A, BCL2, STAT3, AR, EPAS1, BRCA2, or CLU.

Examples of commercial oligonucleotides which can be modified as described herein include, but are not limited to, inclisiran, mipomersen (Kynamro), nusinersen (Spinraza), eteplirsen (Exondys51), miravirsen (SPC3649), RG6042 (IONIS-HTTRx), inotersen, volanesorsen, golodirsen (Vyondys53), fomivirsen (Vitravene), patisiran, givosiran, inclisiran, danvatirsen and IONIS-AR-2.5Rx.

#### 10 Compositions

Oligonucleotides of the disclosure may be admixed, encapsulated, conjugated (such as fused) or otherwise associated with other molecules, molecule structures or mixtures of compounds, resulting in, for example, liposomes, receptor-targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption-assisting formulations include, but are not limited to, US 5,108,921, US 5,354,844, US 5,416,016, US 5,459,127, US 5,521,291, US 5,543,158, US 5,547,932, US 5,583,020, US 5,591,721, US 4,426,330, US 4,534,899, US 5,013,556, US 5,108,921, US 5,213,804, US 5,227,170, US 5,264,221, US 5,356,633, US 5,395,619, US 5,416,016, US 5,417,978, US 5,462,854, US 5,469,854, US 5,512,295, US 5,527,528, US 5,534,259, US 5,543,152, US 5,556,948, US 5,580,575, and US 5,595,756.

Oligonucleotides of the disclosure may be administered in a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may be solid or liquid. Useful examples of pharmaceutically acceptable carriers include, but are not limited to, diluents, solvents, surfactants, excipients, suspending agents, buffering agents, lubricating agents, adjuvants, vehicles, emulsifiers, absorbants, dispersion media, coatings, stabilizers, protective colloids, adhesives, thickeners, thixotropic agents, penetration agents, sequestering agents, isotonic and absorption delaying agents that do not affect the activity of the active agents of the disclosure.

In one embodiment, the pharmaceutical carrier is water for injection (WFI) and the pharmaceutical composition is adjusted to pH 7.4, 7.2-7.6. In one embodiment, the salt is a sodium or potassium salt.

The oligonucleotides may contain chiral (asymmetric) centers or the molecule as a whole may be chiral. The individual stereoisomers (enantiomers and diastereoisomers) and mixtures of these are within the scope of the present disclosure.

Oligonucleotides of the disclosure may be pharmaceutically acceptable salts, esters, or salts of the esters, or any other compounds which, upon administration are capable of providing (directly or indirectly) the biologically active metabolite. The term "pharmaceutically acceptable salts" as used herein refers to physiologically and pharmaceutically acceptable salts of the oligonucleotide that retain the desired biological activities of the parent compounds and do not impart undesired toxicological effects upon administration. Examples of pharmaceutically acceptable salts and their uses are further described in US 6,287,860.

Oligonucleotides of the disclosure may be prodrugs or pharmaceutically acceptable salts of the prodrugs, or other bioequivalents. The term "prodrugs" as used herein refers to therapeutic agents that are prepared in an inactive form that is converted to an active form (i.e., drug) upon administration by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug forms of the oligonucleotide of the disclosure are prepared as SATE [(S acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510, WO 94/26764 and US 5,770,713.

A prodrug may, for example, be converted within the body, e. g. by hydrolysis in the blood, into its active form that has medical effects. Pharmaceutical acceptable prodrugs are described in T. Higuchi and V. Stella, Prodrugs as Novel Delivery Systems, Vol. 14 of the A. C. S. Symposium Series (1976); "Design of Prodrugs" ed. H. Bundgaard, Elsevier, 1985; and in Edward B. Roche, ed., Bioreversible Carriers in Drug Design, American Pharmaceutical Association and Pergamon Press, 1987. Those skilled in the art of organic chemistry will appreciate that many organic compounds can form complexes with solvents in which they are reacted or from which they are precipitated or crystallized. These complexes are known as "solvates". For example, a complex with water is known as a "hydrate".

In one embodiment, oligonucleotides of the invention can be complexed with a complexing agent to increase cellular uptake of oligonucleotides. An example of a complexing agent includes cationic lipids. Cationic lipids can be used to deliver oligonucleotides to cells.

The term "cationic lipid" includes lipids and synthetic lipids having both polar and non-polar domains and which are capable of being positively charged at or around physiological pH and which bind to polyanions, such as nucleic acids, and facilitate the delivery of nucleic acids into cells. In general cationic lipids include saturated and unsaturated alkyl and alicyclic ethers and esters of amines, amides, or derivatives thereof. Straight-chain and branched alkyl and alkenyl groups of cationic lipids can contain, e.g., from 1 to about 25 carbon atoms. Preferred straight chain or branched alkyl or alkene

groups have six or more carbon atoms. Alicyclic groups include cholesterol and other steroid groups. Cationic lipids can be prepared with a variety of counterions (anions) including, e.g., Cl<sup>-</sup>, Br<sup>-</sup>, I<sup>-</sup>, F<sup>-</sup>, acetate, trifluoroacetate, sulfate, nitrite, and nitrate.

Examples of cationic lipids include polyethylenimine, polyamidoamine (PAMAM) starburst dendrimers, Lipofectin (a combination of DOTMA and DOPE), Lipofectase, LIPOFECTAMINE™ (e.g., LIPOFECTAMINE™ 2000), DOPE, Cytofectin (Gilead Sciences, Foster City, Calif.), and Eufectins (JBL, San Luis Obispo, Calif.). Exemplary cationic liposomes can be made from N-[1-(2,3-dioleloxy)-propyl]-N,N,N-trimethylammonium chloride (DOTMA), N-[1-(2,3-dioleloxy)-propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP), 3.beta.-[N--(N',N'-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol), 2,3,-dioleloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; and dimethyldioctadecylammonium bromide (DDAB). Oligonucleotides can also be complexed with, e.g., poly (L-lysine) or avidin and lipids may, or may not, be included in this mixture, e.g., steryl-poly (L-lysine).

Cationic lipids have been used in the art to deliver oligonucleotides to cells (see, e.g., US 5,855,910; 5,851,548; 5,830,430; 5,780,053; 5,767,099; Lewis et al., 1996; Hope et al., 1998). Other lipid compositions which can be used to facilitate uptake of the instant oligonucleotides can be used in connection with the methods of the invention. In addition to those listed above, other lipid compositions are also known in the art and include, e.g., those taught in US 4,235,871; US 4,501,728; 4,837,028; 4,737,323.

In one embodiment lipid compositions can further comprise agents, e.g., viral proteins to enhance lipid-mediated transfections of oligonucleotides. In another embodiment N-substituted glycine oligonucleotides (peptoids) can be used to optimize uptake of oligonucleotides.

In another embodiment, a composition for delivering oligonucleotides of the invention comprises a peptide having from between about one to about four basic residues. These basic residues can be located, e.g., on the amino terminal, C-terminal, or internal region of the peptide. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine (can also be considered non-polar), asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g.,

tyrosine, phenylalanine, tryptophan, histidine). Apart from the basic amino acids, a majority or all of the other residues of the peptide can be selected from the non-basic amino acids, e.g., amino acids other than lysine, arginine, or histidine. Preferably a preponderance of neutral amino acids with long neutral side chains are used.

5 In one embodiment, oligonucleotides are modified by attaching a peptide sequence that transports the oligonucleotide into a cell, referred to herein as a "transporting peptide." In one embodiment, the composition includes an oligonucleotide which is complementary to a target nucleic acid molecule encoding the protein, and a covalently attached transporting peptide.

10 In a further embodiment, the oligonucleotide is attached to a targeting moiety such as N-acetylgalactosamine (GalNAc), an antibody, antibody-like molecule or aptamer (see, for example, Toloue and Ford (2011) and Esposito et al. (2018)).

#### Administration

15 In one embodiment, the oligonucleotide of the disclosure is administered systemically. As used herein "systemic administration" is a route of administration that is either enteral or parenteral.

As used herein "enteral" refers to a form of administration that involves any part of the gastrointestinal tract and includes oral administration of, for example, the  
20 oligonucleotide in tablet, capsule or drop form; gastric feeding tube, duodenal feeding tube, or gastrostomy; and rectal administration of, for example, the oligonucleotide in suppository or enema form.

As used herein "parenteral" includes administration by injection or infusion. Examples include, intravenous (into a vein), intraarterial (into an artery), intramuscular  
25 (into a muscle), intracardiac (into the heart), subcutaneous (under the skin), intraosseous infusion (into the bone marrow), intradermal, (into the skin itself), intrathecal (into the spinal canal), intraperitoneal (infusion or injection into the peritoneum), intravesical (infusion into the urinary bladder), transdermal (diffusion through the intact skin), transmucosal (diffusion through a mucous membrane), inhalational.

30 In one embodiment, administration of the pharmaceutical composition is subcutaneous.

The oligonucleotide may be administered as single dose or as repeated doses on a period basis, for example, daily, once every two days, three, four, five, six seven, eight, nine, ten, eleven, twelve, thirteen or fourteen days, once weekly, twice weekly, three  
35 times weekly, every two weeks, every three weeks, every month, every two months, every three months to six months or every 12 months.

In one embodiment, administration is 1 to 3 times per week, or once every week, two weeks, three weeks, four weeks, or once every two months.

In one embodiment, administration is once weekly.

In one embodiment, a low dose administered for 3 to 6 months, such as about 25-  
5 50mg/week for at least three to six months and then up to 12 months and chronically.

Illustrative doses are between about 10 to 5,000mg. Illustrative doses include 25, 50, 100, 150, 200, 1,000, 2,000mg. Illustrative doses include 1.5mg/kg (about 50 to 100mg) and 3mg/kg (100-200mg), 4.5mg/kg (150-300mg), 10mg/kg, 20mg/kg or 30mg/kg. In one embodiment doses are administered once per week. Thus in one  
10 embodiment, a low dose of approximately 10 to 30, or 20 to 40, or 20 to 28mg may be administered to subjects typically weighing between about 25 and 65kg. In one embodiment the oligonucleotide is administered at a dose of less than 50mg, or less than 30mg, or about 25mg per dose to produce a therapeutic effect.

## 15 **EXAMPLES**

### **EXAMPLE 1 - Methods**

#### **Ethics statement**

Collection of peripheral blood mononuclear cells (PBMCs) from healthy donors was approved by Monash Health under the HREC reference 02052A.

20

#### **Cell isolation, culture and stimulation**

PBMCs were isolated from whole blood donations via density centrifugation using Histopaque-1770 (Sigma-Aldrich) as previously reported (Gantier et al., 2010), and plated in RPMI 1640 plus L-glutamine medium (Thermo Fisher Scientific)  
25 complemented with 1x antibiotic/antimycotic and 10% heat-inactivated foetal bovine serum (referred to as complete RPMI).

293XL-hTLR8-HA (referred to as HEK-TLR8) and 293XL-hTLR7-HA (referred to as HEK-TLR7) and 293XL-hTLR9-HA stably expressing TLR8, TLR7, and TLR9 respectively, were purchased from Invivogen, and were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated foetal bovine serum (Thermo Fisher Scientific) and 1x antibiotic/antimycotic (Thermo Fisher Scientific) (referred to as complete DMEM) supplemented with 10 µg/ml Blasticidin (Invivogen). Parental wild-type (WT) THP-1, UNC93B1- deficient THP-1  
30 (Schmid-Burgk et al., 2014) and matched clones reconstituted with fluorescent wild-type UNC93B1 (Pelka et al., 2014) were grown in complete RPMI. OCI-AML3 and MOLM13 were grown in RPMI supplemented with 20% heat inactivated foetal bovine

serum and 1× antibiotic/antimycotic (their identity was confirmed by in house cell line identification service relying on PowerPlex HS16 System kit, Promega). All the cells were cultured at 37°C with 5% CO<sub>2</sub>. Cell lines were passaged 2-3 times a week and tested for mycoplasma contamination on routine basis by PCR.

- 5 For stimulations, THP-1, MOLM13 and OCI-AML3 were treated overnight with ASOs, prior to stimulation with 1 µg/ml R848 (Invivogen). HEK-TLR7 and HEKTLR8 were treated with indicated concentration of ASOs for 20-50 min, prior to stimulation with R848, CL075, Gardiquimod (all from Invivogen), or 7-Allyl-7,8-dihydro-8-oxoguanosine (Loxoribine – SigmaAldrich). All ASOs were synthesised by Integrated  
 10 DNA Technologies (IDT), and resuspended in RNase-free TE buffer, pH 8.0 (Thermo Fisher Scientific). ASO sequences and modifications are provided in Table 1, 2 and 3. The cGAS ligand ISD70 (Table 1) was prepared as previously described (Pepin et al., 2020) and transfected with lipofectamine 2000 at 2.5 µg/ml final concentration. The Class B CpG oligonucleotide human TLR9 ligand ODN 2006 was synthesised by IDT  
 15 and resuspended in RNase-free TE buffer

#### Luciferase assays

- HEK293 cells stably expressing TLR7, 8 or 9 were transfected with pNF-κB-Luc4 reporter (Clontech), pLuc-IFN-β (a kind gift from K. Fitzgerald, University of  
 20 Massachusetts) or pCCL5[RANTES]-Luc (a kind gift from G. Scholz, University of Melbourne) with lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's protocol. Briefly, 500,000-700,000 cells were reverse-transfected with 400 ng of reporter with 1.2 µl of lipofectamine 2000 per well of a 6-well plate, and incubated for 3-24 h at 37°C with 5% CO<sub>2</sub>. Following transfection, the cells were  
 25 collected from the 6-wells and aliquoted into 96-wells, just before ASO and overnight TLR stimulation (as above described). The next day, the cells were lysed in 40 µl (for a 96-well plate) of 1X Glo Lysis buffer (Promega) for 10 min at room temperature. 15 µl of the lysate was then subjected to firefly luciferase assay using 40 µl of Luciferase Assay Reagent (Promega). Luminescence was quantified with a Fluostar OPTIMA (BMG  
 30 LABTECH) luminometer.

#### Down-regulation of HPRT with ASOs in HeLa cells

- Each ASO was reverse-transfected in biological triplicate in 96-well plates by complexing the various ASO doses with 0.5 µl Lipofectamine 2000 (Thermo Fisher  
 35 Scientific) in OptiMEM I (Thermo Fisher Scientific) for a total volume of 50 µl in each well. HeLa cells (20,000) were suspended in 100 µl DMEM supplemented with 10%

foetal calf serum (FCS), added to the lipid-ASO complexes, then incubated for 24 h at 37°C and 5% CO<sub>2</sub>. RNA was collected with the SV Total RNA Isolation Kit (Promega) with DNaseI treatment. cDNA was synthesized from ~200 ng total RNA with anchored oligonucleotide dT and random hexamer primers (Integrated DNA Technologies) using  
 5 SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) as per the manufacturer's instructions. qPCR reactions were performed using ~10 ng cDNA with Immolase DNA polymerase (Bioline), 500 nM of each primer and 250 nM probe in 10 µl reactions in 384-well plate format. Amplification reactions were run on an Applied Biosystems 7900HT (Thermo Fisher Scientific). All qPCR reactions were performed in  
 10 triplicate for each sample and averaged.

Linearized cloned amplicons were used as copy number standards to establish absolute quantitative measurements for each assay. HPRT (NM 000194) and SFRS9 (NM 003769) expression levels were quantified by multiplexing 5'-nuclease assays, and HPRT levels normalized against SFRS9 – used as internal reference control. Sequences  
 15 of the primers and probe assays used are provided in Table 1. Knock-down efficiency was calculated relative to NC1 and NC5 negative control ASOs.

#### Detection of cytokines

Human TNF-α and IP-10 were measured using BD OptEIA ELISA sets (BD  
 20 Biosciences, #555212 and #550926, respectively), according to the manufacturers' instructions. Human IFN-α detection was carried out as previously reported (Gantier, 2013). Tetramethylbenzidine substrate (Thermo Fisher Scientific) was used for quantification of the cytokines on a Fluostar OPTIMA (BMG LABTECH) plate-reader.

#### mRNA reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was purified from cells using the ISOLATE II RNA Mini Kit (Bioline). Random hexamer cDNA was synthesized from isolated RNA using the High-Capacity cDNA Archive kit (Thermo Fisher Scientific) according to the manufacturer's instructions. RT-qPCR was carried out with the Power SYBR Green Master Mix  
 30 (Thermo Fisher Scientific) on the HT7900 and QuantStudio 6 RT-PCR system (Thermo Fisher Scientific). Each PCR was carried out in technical duplicate and human 18S was used as reference gene. Each amplicon was gel-purified and used to generate a standard curve for the quantification of gene expression (used in each run). Melting curves were used in each run to confirm specificity of amplification.

35 The primers used were the following: Human RSAD2: hRSAD2-RT-FWD  
 TGGTGAGGTTCTGCAAAGTAG; hRSAD2-RT-REV

GTCACAGGAGATAGCGAGAATG; hIFIT1: hIFIT1-FWD  
 TCACCAGATAGGGCTTTGCT; hIFIT1-REV CACCTCAAATGTGGGCTTTT;  
 h18S: h18S-FWD CGGCTACCACATCCAAGGAA; h18S-REV  
 GCTGGAATTACCGCGGCT; hIFI44: hIFI44-FWD  
 5 ATGGCAGTGACAACTCGTTTG; hIFI44: TCCTGGTAACTCTCTTCTGCATA;  
 hIFNB: hIFNB-FWD GCTTGGATTCTTACAAAGAAGCA; hIFNBREV:  
 ATAGATGGTCAATGCGGCGTC; hHPRT-FWD: GACTTTGCTTTCCTTGGTCAG;  
 hHPRT-REV GGCTTATATCCAACACTTCGTGGG; amplicons from RSAD2, IFIT1,  
 and 18S PCRs were verified by Sanger sequencing. IFI44 and IFNB primers were from  
 10 the Primer Bank (Wang et al., 2012), and HPRT primers were designed by IDT.

#### Statistical analyses

Statistical analyses were carried out using Prism 8 (GraphPad Software Inc.).  
 15 Every experiment was carried out in biological triplicate (except Figures 2A, 2B, 4D and  
 5E, carried out in biological duplicate) and repeated a minimum of two independent  
 times. One-way and two-way analyses of variance (ANOVA) were used when comparing  
 groups of conditions, while two-tailed unpaired non-parametric Mann-Whitney U tests  
 or unpaired two-tailed t-tests were used when comparing pairs of conditions. Symbols  
 20 used: \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , \*\*\*\*  $P \leq 0.0001$  and “ns” is non-significant.

#### **EXAMPLE 2: Sequence and backbone-dependent effects of ASOs on TLR7/8 sensing**

The inventors initially investigated the activity of a panel of 11 2'OMe gapmer  
 25 ASOs targeted to the mRNA of the innate immune sensor cGAS, on immune responses  
 of undifferentiated THP-1 cells. Surprisingly, overnight pre-treatment with the ASOs led  
 to strong potentiation of IP-10 and TNF- $\alpha$  production upon R848 stimulation of TLR7/8  
 in the cells, for select ASOs (e.g. ASO2, ASO9, ASO11, but not ASO4 - Figure 1A).  
 Previous studies have reported that T-rich PS oligonucleotides could promote TLR8  
 30 sensing, while inhibiting TLR7 (Gorden et al., 2006; Jurk et al., 2006). Since THP-1 can  
 respond to both TLR7 and TLR8 ligands (Gantier et al., 2008), the inventors speculated  
 that the sequence-specific effect of the ASOs on R848 sensing they observed could be  
 due to their different activities on TLR7 and TLR8. To define this, the inventors next  
 tested our panel of sequences in HEK 293 cells stably expressing TLR7 or TLR8  
 35 (referred to as HEK-TLR7 and HEKTLR8 hereafter), along with an NF- $\kappa$ B-luciferase  
 reporter (Figures 1B, 1C, 8A and 8B).

Interestingly, the inventors found that most ASOs strongly inhibited TLR7 sensing of R848, with the exception of ASO8 and ASO11, which were less potent inhibitors (Figure 1B). Conversely, and directly aligning with the inventors observations in THP-1 cells, several ASOs strongly potentiated TLR8 sensing of R848 (e.g. ASO2, ASO9 and ASO11 - Figure 1C). The ASOs on their own did not stimulate TLR7 or TLR8 (Figures 8A, 8B). Focusing on ASO2 and ASO11, which equally potentiated TLR8 but had different activities on TLR7, the inventors validated further their TLR7/8-dependent activity in THP-1 lacking UNC93B1, which is essential to TLR7/8 signalling (Pelka et al., 2014). The potentiation effect of ASO2 and ASO11 on R848 sensing was not seen in THP-1 lacking UNC93B1, but could be restored upon reconstitution of UNC93B1-Citrine expression (Pelka et al., 2014) (Figure 1D), thereby supporting the involvement of TLR7/8 in this effect. In addition, stimulation of human peripheral blood mononuclear cells (PBMCs) with ASO2 and ASO11 strongly potentiated R848 induced TNF- $\alpha$ , but did not impact IFN- $\alpha$  levels, indicative of a preferential effect on TLR8 sensing of R848 (Gantier et al., 2008) (Figures 1E, 1F). This effect on IFN- $\alpha$  and TNF- $\alpha$  was not related to TLR9 activation of PBMCs by the ASOs, since ASO2 did not activate TLR9 signalling in HEK-TLR9 cells, while ASO11 did (Figure 8C).

To define whether this effect of the ASOs on TLR7/8 was dependent on their backbone or base modifications, the inventors next studied a series of ASO variants based on the sequence of ASO2 (Figures 1G, 1H, 1I). Analyses of these variants in HEK-TLR7 cells stimulated with R848 revealed that all ASO2 variants containing a PS backbone inhibited TLR7, independent of the type of base modifications used (DNA only, 2'OMe, LNA or 2'MOE) (Figures 1G, 1H, Table 1). Conversely, potentiation of R848 sensing by TLR8 was directly dependent on the 5' and 3' end base modifications, with 2'OMe giving the best potentiation in this sequence context (Figures 1G, 1I). Addition of a 3' end Cy3 linker decreased this potentiation of TLR8 sensing, while substitution of 2'OMe bases with 2'MOE or LNA bases ablated the potentiation.

Potentiation was not limited to the dual TLR7/8 agonist R848 and was also seen with CL075 (TLR8 agonist), Loxoribine (TLR7 agonist), and to some extent with Gardiquimod (TLR7 agonist) (Figure 7).

Similar to the effect observed on TLR7, the PS backbone was also necessary for TLR8 potentiation; it was not, however, sufficient for this effect by itself, since the 2'OMe and LNA ASO2 variants were also synthesised on a PS backbone, and only limited potentiation was seen with the variant featuring the PS modification only (ASO2-PS). Collectively these results demonstrated that PS ASOs could display potent TLR7/8 immunomodulation, in a sequence-dependent manner.

**Table 1:** Various oligonucleotides used in this study (all in 5'-3'). UPPERCASE alone for DNA, 'm' indicates 2'OMe base, '/i2MOEr' indicates 2'MOE base, '+' indicates LNA base, and \* denotes the phosphorothioate backbone. Cy3Sp denotes the Cy3 tag. FAM, HEX, ZEN, 3IABkFQ are qPCR probe modifications.

5

| Name                             | Sequence                                               | SEQ ID NO. |
|----------------------------------|--------------------------------------------------------|------------|
| NC1 2OMe/PS<br>ASO<br>(Neg Cont) | mG*mC*mG*mU*mA*T*T*A*T*A*G*C*C*G*A*mU*mU*mA*<br>mA*mC  | 1          |
| NC5 2OMe/PS<br>ASO<br>(Neg Cont) | mG*mC*mG*mA*mC*T*A*T*A*C*G*C*G*C*A*mA*mU*mA*<br>mU*mG  | 2          |
| [cGAS]ASO1                       | mA*mU*mG*mG*mC*C*T*T*T*C*C*G*T*G*C*mC*mA*mA*m<br>G*mG  | 3          |
| [cGAS]ASO2                       | mU*mC*mC*mG*mG*C*C*T*C*G*G*A*A*G*C*mU*mC*mU*<br>mC*mU  | 4          |
| [cGAS]ASO3                       | mG*mC*mA*mU*mU*C*C*G*T*G*C*G*G*A*A*mG*mC*mC*<br>mU*mU  | 5          |
| [cGAS]ASO4                       | mG*mG*mC*mC*mG*A*A*C*T*T*T*C*C*G*mC*mC*mU*<br>mU*mA    | 6          |
| [cGAS]ASO5                       | mG*mG*mU*mC*mU*T*G*G*C*T*T*C*G*T*G*mG*mA*mG*<br>mC*mA  | 7          |
| [cGAS]ASO6                       | mG*mG*mA*mG*mC*T*T*C*G*A*G*G*C*C*C*mC*mA*mG*<br>mG*mC  | 8          |
| [cGAS]ASO7                       | mG*mG*mU*mG*mG*T*C*C*A*C*A*A*C*C*C*mC*mU*mU*<br>mU*mC  | 9          |
| [cGAS]ASO8                       | mC*mA*mU*mU*mA*G*G*T*G*C*A*G*A*A*A*mU*mC*mU*<br>*mU*mC | 10         |
| [cGAS]ASO9                       | mU*mU*mC*mU*mG*G*G*A*C*T*T*T*C*A*mG*mU*mU*<br>*mU*mA   | 11         |

|              |                                                                                                                          |    |
|--------------|--------------------------------------------------------------------------------------------------------------------------|----|
| [cGAS]ASO10  | mU*mG*mA*mU*mU*C*C*A*A*A*G*C*A*G*mG*mG*mU<br>*mU*mA                                                                      | 12 |
| [cGAS]ASO11  | mC*mU*mU*mU*mA*G*T*C*G*T*A*G*T*T*G*mC*mU*mU*<br>mC*mC                                                                    | 13 |
| ASO2-Cy3     | mU*mC*mC*mG*mG*C*C*T*C*G*G*A*A*G*C*mU*mC*mU*<br>mC*mU/3Cy3Sp/                                                            | 14 |
| ASO2-PS      | T*C*C*G*G*C*C*T*C*G*G*A*A*G*C*T*C*T*C*T                                                                                  | 15 |
| ASO2-PO      | mUmCmCmGmGCCTCGGAAGCmUmCmUmCmU                                                                                           | 16 |
| ASO2-LNA     | +C*+G*+G*C*C*T*C*G*G*A*A*G*C*+T*+C*+T                                                                                    | 17 |
| ASO2-2MOE    | /52MOErT/*i2MOErC/*i2MOErC/*i2MOErG/*i2MOErG/*C*C<br>*T*C*G*G*A*A*G*C/*i2MOErT/*i2MOErC/*<br>/i2MOErT/*i2MOErC/*32MOErT/ | 18 |
| ASO11-Mut1   | mC*mU*mU*mU*mA*G*T*C*G*T*A*G*T*T*G*mU*mC*mU*<br>mC*mU                                                                    | 19 |
| ASO11-Mut2   | mU*mC*mC*mG*mG*G*T*C*G*T*A*G*T*T*G*mC*mU*mU*<br>mC*mC                                                                    | 20 |
| ASO852       | mC*mU*mC*mU*mC*T*T*T*C*T*G*T*G*G*T*mU*mU*mC*<br>mU*mC                                                                    | 21 |
| ASO852-dT    | mC*mU*mC*mU*mC*T*T*T*T*T*T*T*T*T*mU*mU*mC*m<br>U*mC                                                                      | 22 |
| ASO2504      | mC*mC*mU*mA*mU*T*A*A*A*A*A*A*T*T*mU*mA*mU<br>*mA*mC                                                                      | 23 |
| ASO2504-Mut  | mC*mC*mU*mA*mU*T*T*T*C*T*G*T*G*G*T*mU*mA*mU*<br>mA*mC                                                                    | 24 |
| dT20         | T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T                                                                                  | 25 |
| Hs HPRT F517 | GACTTTGCTTTCCTTGGTCAG                                                                                                    | 26 |

|                      |                                                                                                  |    |
|----------------------|--------------------------------------------------------------------------------------------------|----|
| Hs HPRT R591         | GGCTTATATCCAACACTTCGTGGG                                                                         | 27 |
| Hs HPRT P554<br>FAM  | /56-<br>FAM/ATGGTCAAG/ZEN/GTCGCAAGCTTGCTGGT/3IABkFQ/                                             | 28 |
| Hs SFRS9 F594        | GTCGAGTATCTCAGAAAAGAAGACA                                                                        | 29 |
| Hs SFRS9 R690        | CTCGGATGTAGGAAGTTTCACC                                                                           | 30 |
| Hs SFRS9 P625<br>HEX | /5HEX/ATGCCCTGC/ZEN/GTAACTGGATGACA/3IABkFQ/                                                      | 31 |
| ODN 2006             | T*C*G* T*C*G* T*T*T* T*G*T* C*G*T* T*T*T* G*T*C*<br>G*T*T                                        | 32 |
| ISD70-FWD            | CCA TCA GAA AGA GGT TTA ATA TTT TTG TGA GAC CAT<br>CGA AGA GAG AAA GAG ATA AAA CTT TTT TAC GAC T | 33 |
| ISD70-REV            | AGT CGT AAA AAA GTT TTA TCT CTT TCT CTC TTC GAT<br>GGT CTC ACA AAA ATA TTA AAC CTC TTT CTG ATG G | 34 |

**EXAMPLE 3: Screen to identify ASOs with low TLR7 inhibition and high TLR8 potentiation**

The observation of TLR7 inhibition and TLR8 potentiation by select PS ASOs aligned with the previous reports that T-rich PS oligonucleotides could promote similar activities (Gorden et al., 2006; Jurk et al., 2006). However, the finding that some 2'OMe ASO sequences had less inhibitory activity on TLR7 (e.g. ASO8 and ASO11) suggested that TLR7 inhibition promoted by the PS backbone may be counterbalanced in select 2'OMe gapmer ASOs. The inventors reasoned that defining the modalities of this activity could help design ASOs with reduced immunosuppressive activities towards TLR7. In addition, the observation that ASO11 was able to potentiate TLR8 sensing of R848 while preserving TLR7 activity, suggested that the activities on TLR7 and 8 were not governed by the same sequence determinants.

To characterize these observations further, the inventors screened a library of 192 2'OMe ASOs. It is noteworthy that these ASOs were designed to target 4 different transcripts (48 ASOs each), with a minimum of single base increments between the ASOs (Table 2). The screen was performed at two different ASO concentrations for each TLR

and measured their impact on NF- $\kappa$ B luciferase induction by R848 in HEK-TLR7 and HEK-TLR8 cells (Figures 2A, 2B and Table 2).

In agreement with the initial panel of ASOs, the inventors found that the majority of ASOs used at 500 nM strongly suppressed TLR7 sensing. As such, 78% of the ASOs  
5 reduced R848 activity on TLR7 by more than 80%, and only 2 ASOs reduced TLR7 sensing by less than 40% at both concentrations (Figure 2A and Table 2 – ASOs ‘[CDKN2B-AS1]-852’ and ‘[LINC-PINT]-2504’, referred to as ASO852 and ASO2504 hereafter). Conversely, the effect of ASOs on TLR8 potentiation was diverse across ASOs with 51% of the ASOs potentiating R848 sensing by at least 2 fold at 100 nM  
10 (Figure 2B). Importantly, while both displaying low TLR7 inhibition, ASO852 and ASO2504 had distinct activities on TLR8 (Figure 2A, 2B and Table 2). ASO dose-response studies in HEK293-TLR7 and HEK293-TLR8 cells confirmed that ASO2504 and ASO852 had little impact on R848 sensing by TLR7 compared to ASO4, however ASO852 potentiated TLR8 sensing significantly more than ASO4 (Figure 2C). Analyses  
15 of the impact of ASO852 on various doses of R848 revealed that it decreased the sensitivity of TLR7 to R848 by ~2.5 fold (Figure 2D). However, ASO852 treatment increased the activity of R848 on TLR8 ~13 fold (Figure 2D).

**Table 2:** 196 ASO screen data. The targeted gene names are provided in brackets (e.g. [EGFR]). ASOs were synthesised with the following modifications: UPPERCASE alone for DNA, 'm' indicates 2'OMe base modifications, and \*denotes the phosphorothioate backbone. Averaged NF- $\kappa$ B- Luciferase data from each screen at indicated ASO concentration is given (using 1 $\mu$ g/ml R848 co-stimulation). Underlined sequences from the PINT family were studied further in Figure 3B and 3F. Sequences in black bold were used as 17 low TLR7 inhibitors and analysed with MEME for motif enrichment (along with [cGAS]ASO8 and ASO11) shown in Figure 3A.

| [Transcript Name] and Reference | Sequence                                                  | TLR8-500nM | TLR8-100nM | TLR7-500nM | TLR7-100nM | SEQ ID NO. |
|---------------------------------|-----------------------------------------------------------|------------|------------|------------|------------|------------|
| [CDKN2B-AS1]1240                | mG*mU*mC*mU*mC*T*<br>A*C*T*G*T*T*A*C*C*m<br>U*mC*mU*mG*mA | 5.79       | 2.41       | 16.16      | 19.16      | 35         |
| [CDKN2B-AS1]132                 | mU*mU*mA*mA*mA*T*<br>A*A*T*C*T*A*G*T*T*m<br>U*mG*mA*mA*mG | 6.43       | 3.96       | 15.33      | 25.66      | 36         |
| [CDKN2B-AS1]1415                | mG*mU*mG*mU*mC*C*<br>T*T*C*A*T*G*C*T*T*m<br>U*mG*mG*mA*mU | 1.60       | 1.33       | 12.24      | 18.05      | 37         |
| [CDKN2B-AS1]1519                | mA*mG*mA*mA*mA*G*<br>A*A*G*C*A*A*A*G*A*<br>mU*mU*mC*mA*mA | 2.78       | 1.96       | 17.19      | 42.99      | 38         |
| [CDKN2B-AS1]1522                | mC*mC*mU*mA*mG*A*<br>A*A*G*A*A*G*C*A*A*<br>mA*mG*mA*mU*mU | 4.94       | 3.58       | 24.37      | 57.20      | 39         |
| [CDKN2B-AS1]1528                | mG*mU*mC*mA*mA*A*<br>C*C*T*A*G*A*A*A*G*<br>mA*mA*mG*mC*mA | 1.85       | 1.60       | 16.51      | 23.64      | 40         |
| [CDKN2B-AS1]1773                | mG*mA*mU*mU*mA*A*<br>A*A*C*A*G*A*T*T*A*<br>mA*mU*mA*mC*mA | 1.48       | 0.73       | 15.20      | 21.92      | 41         |
| [CDKN2B-AS1]1774                | mG*mG*mA*mU*mU*A*<br>A*A*A*C*A*G*A*T*T*<br>mA*mA*mU*mA*mC | 1.67       | 1.16       | 14.92      | 27.16      | 42         |

|                  |                                                                                 |             |             |              |              |    |
|------------------|---------------------------------------------------------------------------------|-------------|-------------|--------------|--------------|----|
| [CDKN2B-AS1]1775 | mA*mG*mG*mA*mU*T*<br>A*A*A*A*C*A*G*A*T*<br>mU*mA*mA*mU*mA                       | 2.63        | 2.19        | 58.42        | 36.28        | 43 |
| [CDKN2B-AS1]2108 | mG*mA*mG*mU*mU*C*<br>T*T*C*G*T*A*G*G*C*m<br>U*mU*mC*mU*mG                       | 1.23        | 0.96        | 15.37        | 25.86        | 44 |
| [CDKN2B-AS1]2130 | mA*mG*mA*mU*mU*A*<br>T*C*T*T*C*T*T*T*T*m<br>A*mA*mU*mU*mU                       | 6.26        | 3.02        | 19.91        | 37.10        | 45 |
| [CDKN2B-AS1]2131 | mA*mA*mG*mA*mU*T*<br>A*T*C*T*T*C*T*T*T*m<br>U*mA*mA*mU*mU                       | 5.62        | 2.91        | 20.16        | 42.09        | 46 |
| [CDKN2B-AS1]2132 | mA*mA*mA*mG*mA*T*<br>T*A*T*C*T*T*C*T*T*m<br>U*mU*mA*mA*mU                       | 5.04        | 2.96        | 24.62        | 49.28        | 47 |
| [CDKN2B-AS1]2133 | mA*mA*mA*mA*mG*A*<br>T*T*A*T*C*T*T*C*T*m<br>U*mU*mU*mA*mA                       | 4.68        | 3.19        | 20.33        | 47.41        | 48 |
| [CDKN2B-AS1]2134 | mG*mA*mA*mA*mA*G*<br>A*T*T*A*T*C*T*T*C*m<br>U*mU*mU*mU*mA                       | 1.87        | 1.31        | 18.57        | 34.44        | 49 |
| [CDKN2B-AS1]2137 | mU*mG*mU*mG*mA*A*<br>A*A*G*A*T*T*A*T*C*m<br>U*mU*mC*mU*mU                       | 4.97        | 2.79        | 18.34        | 34.51        | 50 |
| [CDKN2B-AS1]2138 | mU*mU*mG*mU*mG*A*<br>A*A*A*G*A*T*T*A*T*m<br>C*mU*mU*mC*mU                       | 1.94        | 1.05        | 19.49        | 35.02        | 51 |
| [CDKN2B-AS1]2139 | <b>mC*mU*mU*mG*mU*G*</b><br><b>*A*A*A*A*G*A*T*T*A*</b><br><b>mU*mC*mU*mU*mC</b> | <b>6.02</b> | <b>3.89</b> | <b>49.18</b> | <b>85.33</b> | 52 |
| [CDKN2B-AS1]2196 | mG*mG*mU*mG*mG*C*<br>C*A*C*A*G*G*C*A*A*<br>mC*mG*mU*mC*mA                       | 0.98        | 0.80        | 16.65        | 39.07        | 53 |
| [CDKN2B-AS1]2198 | mA*mA*mG*mG*mU*G*<br>G*C*C*A*C*A*G*G*C*<br>mA*mA*mC*mG*mU                       | 0.78        | 0.58        | 14.95        | 57.85        | 54 |

|                  |                                                           |      |      |       |       |    |
|------------------|-----------------------------------------------------------|------|------|-------|-------|----|
| [CDKN2B-AS1]2218 | mA*mG*mG*mC*mC*T*<br>C*C*A*G*T*G*T*C*T*m<br>U*mC*mU*mC*mC | 4.66 | 2.19 | 21.33 | 44.73 | 55 |
| [CDKN2B-AS1]2219 | mC*mA*mG*mG*mC*C*<br>T*C*C*A*G*T*G*T*C*m<br>U*mU*mC*mU*mC | 3.89 | 2.02 | 23.06 | 54.11 | 56 |
| [CDKN2B-AS1]2223 | mG*mU*mC*mC*mC*A*<br>G*G*C*C*T*C*C*A*G*m<br>U*mG*mU*mC*mU | 2.17 | 1.05 | 12.54 | 19.01 | 57 |
| [CDKN2B-AS1]2227 | mC*mC*mA*mU*mG*T*<br>C*C*C*A*G*G*C*C*T*m<br>C*mC*mA*mG*mU | 1.46 | 1.24 | 17.53 | 41.39 | 58 |
| [CDKN2B-AS1]2230 | mU*mC*mU*mC*mC*A*<br>T*G*T*C*C*C*A*G*G*m<br>C*mC*mU*mC*mC | 9.17 | 3.41 | 21.16 | 43.45 | 59 |
| [CDKN2B-AS1]2231 | mG*mU*mC*mU*mC*C*<br>A*T*G*T*C*C*C*A*G*m<br>G*mC*mC*mU*mC | 1.25 | 1.01 | 16.91 | 23.75 | 60 |
| [CDKN2B-AS1]2232 | mA*mG*mU*mC*mU*C*<br>C*A*T*G*T*C*C*C*A*m<br>G*mG*mC*mC*mU | 2.28 | 1.64 | 14.11 | 35.77 | 61 |
| [CDKN2B-AS1]2233 | mC*mA*mG*mU*mC*T*<br>C*C*A*T*G*T*C*C*C*m<br>A*mG*mG*mC*mC | 3.81 | 2.73 | 16.62 | 47.75 | 62 |
| [CDKN2B-AS1]2234 | mG*mC*mA*mG*mU*C*<br>T*C*C*A*T*G*T*C*C*m<br>C*mA*mG*mG*mC | 2.25 | 1.79 | 13.30 | 20.30 | 63 |
| [CDKN2B-AS1]2235 | mA*mG*mC*mA*mG*T*<br>C*T*C*C*A*T*G*T*C*m<br>C*mC*mA*mG*mG | 4.93 | 2.81 | 14.78 | 25.50 | 64 |
| [CDKN2B-AS1]2236 | mA*mA*mG*mC*mA*G*<br>T*C*T*C*C*A*T*G*T*m<br>C*mC*mC*mA*mG | 2.62 | 1.79 | 15.46 | 35.69 | 65 |
| [CDKN2B-AS1]2237 | mA*mA*mA*mG*mC*A*<br>G*T*C*T*C*C*A*T*G*m<br>U*mC*mC*mC*mA | 3.38 | 1.90 | 15.95 | 27.16 | 66 |

|                 |                                                           |      |      |       |       |    |
|-----------------|-----------------------------------------------------------|------|------|-------|-------|----|
| [CDKN2B-AS1]368 | mG*mU*mC*mG*mU*G*<br>G*C*A*A*A*T*A*G*T*<br>mC*mC*mU*mA*mG | 3.24 | 2.12 | 14.09 | 27.52 | 67 |
| [CDKN2B-AS1]442 | mG*mG*mA*mG*mA*T*<br>C*A*G*A*T*G*A*G*A*<br>mG*mG*mA*mG*mC | 1.67 | 1.05 | 15.77 | 26.34 | 68 |
| [CDKN2B-AS1]495 | mA*mG*mU*mG*mG*C*<br>A*C*A*T*A*C*C*A*C*m<br>A*mC*mC*mC*mU | 4.48 | 2.24 | 16.83 | 41.95 | 69 |
| [CDKN2B-AS1]568 | mC*mU*mU*mC*mA*C*<br>A*T*C*C*A*A*G*A*C*<br>mA*mG*mC*mA*mA | 7.23 | 2.26 | 25.10 | 55.01 | 70 |
| [CDKN2B-AS1]611 | mG*mU*mG*mU*mU*T*<br>T*T*A*A*T*T*T*G*m<br>U*mA*mG*mA*mG   | 1.88 | 1.31 | 18.82 | 23.63 | 71 |
| [CDKN2B-AS1]613 | mC*mA*mG*mU*mG*T*<br>T*T*T*T*A*A*T*T*T*m<br>U*mG*mU*mA*mG | 6.73 | 3.18 | 45.63 | 62.68 | 72 |
| [CDKN2B-AS1]626 | mA*mU*mU*mU*mC*C*<br>A*C*A*T*G*C*C*A*m<br>G*mU*mG*mU*mU   | 4.09 | 2.06 | 16.15 | 32.05 | 73 |
| [CDKN2B-AS1]627 | mU*mA*mU*mU*mU*C*<br>C*A*C*A*T*G*C*C*m<br>A*mG*mU*mG*mU   | 3.87 | 2.77 | 15.14 | 30.54 | 74 |
| [CDKN2B-AS1]645 | mA*mA*mU*mU*mU*A*<br>A*A*G*C*A*T*G*A*A*<br>mU*mA*mU*mU*mA | 5.02 | 3.29 | 21.11 | 46.75 | 75 |
| [CDKN2B-AS1]79  | mA*mA*mA*mA*mU*A*<br>A*G*G*G*G*A*A*T*A*<br>mG*mG*mG*mG*mA | 0.71 | 0.86 | 19.79 | 68.45 | 76 |
| [CDKN2B-AS1]80  | mU*mA*mA*mA*mA*T*<br>A*A*G*G*G*G*A*A*T*<br>mA*mG*mG*mG*mG | 1.30 | 1.28 | 20.64 | 40.95 | 77 |
| [CDKN2B-AS1]831 | mA*mU*mA*mU*mC*T*<br>G*C*T*G*C*C*A*C*m<br>C*mU*mU*mC*mU   | 5.89 | 2.55 | 18.61 | 47.52 | 78 |

|                 |                                                            |       |      |       |        |    |
|-----------------|------------------------------------------------------------|-------|------|-------|--------|----|
| [CDKN2B-AS1]832 | mA*mA*mU*mA*mU*C*<br>T*G*C*T*G*C*C*A*m<br>C*mC*mU*mU*mC    | 4.45  | 1.79 | 20.07 | 43.66  | 79 |
| [CDKN2B-AS1]852 | mC*mU*mC*mU*mC*T*<br>T*T*C*T*G*T*G*G*T*<br>mU*mU*mC*mU*mC  | 12.67 | 3.82 | 61.82 | 92.33  | 80 |
| [CDKN2B-AS1]924 | mG*mU*mG*mG*mU*T*<br>A*A*G*T*A*C*A*T*G*<br>mA*mG*mC*mU*mC  | 0.83  | 0.59 | 15.50 | 21.58  | 81 |
| [CDKN2B-AS1]965 | mG*mG*mA*mC*mA*C*<br>T*T*A*G*C*T*G*T*T*m<br>C*mC*mU*mC*mG  | 2.62  | 1.13 | 20.92 | 45.77  | 82 |
| [CTNNB1]1212    | mG*mG*mG*mU*mC*C*<br>A*C*C*A*C*T*A*G*C*m<br>C*mA*mG*mU*mA  | 1.95  | 1.18 | 1.57  | 33.47  | 83 |
| [CTNNB1]1234    | mU*mC*mA*mU*mU*A*<br>T*A*T*T*A*C*T*A*m<br>A*mA*mG*mC*mU    | 7.84  | 4.22 | 6.03  | 65.47  | 84 |
| [CTNNB1]1235    | mC*mU*mC*mA*mU*T*<br>A*T*A*T*T*A*C*T*<br>mA*mA*mA*mG*mC    | 7.60  | 3.46 | 5.53  | 93.53  | 85 |
| [CTNNB1]1294    | mC*mA*mG*mA*mU*A*<br>*G*C*A*C*C*T*T*C*A*<br>mG*mC*mA*mC*mU | 5.89  | 3.22 | 4.55  | 126.50 | 86 |
| [CTNNB1]1445    | mU*mC*mC*mA*mU*C*<br>*C*C*T*T*C*C*T*G*T*<br>mU*mU*mA*mG*mU | 7.84  | 4.57 | 6.21  | 96.37  | 87 |
| [CTNNB1]1548    | mC*mU*mU*mA*mU*A*<br>A*T*T*A*T*T*G*C*A*m<br>A*mG*mU*mG*mA  | 6.45  | 2.78 | 4.61  | 74.91  | 88 |
| [CTNNB1]1549    | mU*mC*mU*mU*mA*T*<br>A*A*T*T*A*T*T*G*C*<br>mA*mA*mG*mU*mG  | 7.36  | 4.58 | 5.97  | 110.24 | 89 |
| [CTNNB1]1575    | mA*mC*mC*mC*mA*C*<br>T*T*G*G*C*A*G*A*C*m<br>C*mA*mU*mC*mA  | 6.80  | 2.74 | 4.77  | 65.83  | 90 |

|                  |                                                           |      |      |      |       |     |
|------------------|-----------------------------------------------------------|------|------|------|-------|-----|
| [CTNNB1]15<br>76 | mC*mA*mC*mC*mC*A<br>*C*T*T*G*G*C*A*G*A<br>*mC*mC*mA*mU*mC | 3.00 | 1.12 | 2.06 | 91.47 | 91  |
| [CTNNB1]15<br>77 | mC*mC*mA*mC*mC*C*<br>A*C*T*T*G*G*C*A*G*m<br>A*mC*mC*mA*mU | 2.23 | 0.99 | 1.61 | 86.93 | 92  |
| [CTNNB1]15<br>78 | mA*mC*mC*mA*mC*C*<br>C*A*C*T*T*G*G*C*A*m<br>G*mA*mC*mC*mA | 5.55 | 1.71 | 3.63 | 63.40 | 93  |
| [CTNNB1]16<br>42 | mU*mG*mG*mC*mA*G*<br>G*C*T*C*A*G*T*G*A*m<br>U*mG*mU*mC*mU | 3.26 | 1.73 | 2.49 | 50.44 | 94  |
| [CTNNB1]16<br>78 | mC*mU*mU*mG*mG*T*<br>G*T*C*G*G*C*T*G*G*m<br>U*mC*mA*mG*mA | 3.77 | 2.15 | 2.96 | 79.82 | 95  |
| [CTNNB1]16<br>92 | mG*mG*mC*mC*mA*T*<br>C*T*C*T*G*C*T*T*C*m<br>U*mU*mG*mG*mU | 0.86 | 0.82 | 0.84 | 35.92 | 96  |
| [CTNNB1]17<br>03 | mA*mC*mU*mG*mC*A*<br>T*T*C*T*G*G*G*C*C*m<br>A*mU*mC*mU*mC | 5.59 | 2.96 | 4.28 | 73.17 | 97  |
| [CTNNB1]20<br>71 | mC*mA*mA*mU*mG*G*<br>G*A*G*A*A*T*A*A*A*<br>mG*mC*mA*mG*mC | 5.40 | 2.50 | 3.95 | 73.67 | 98  |
| [CTNNB1]20<br>72 | mU*mC*mA*mA*mU*G*<br>G*G*A*G*A*A*T*A*A*<br>mA*mG*mC*mA*mG | 7.05 | 3.97 | 5.51 | 68.89 | 99  |
| [CTNNB1]20<br>73 | mU*mU*mC*mA*mA*T*<br>G*G*G*A*G*A*A*T*A*<br>mA*mA*mG*mC*mA | 6.41 | 3.03 | 4.72 | 32.94 | 100 |
| [CTNNB1]21<br>36 | mU*mU*mC*mU*mG*C*<br>A*G*C*T*T*C*C*T*T*m<br>G*mU*mC*mC*mU | 7.56 | 4.06 | 5.81 | 42.72 | 101 |
| [CTNNB1]22<br>52 | mU*mG*mU*mG*mG*C*<br>T*T*G*T*C*C*T*C*A*m<br>G*mA*mC*mA*mU | 3.61 | 1.57 | 2.59 | 47.10 | 102 |

|                  |                                                                                |             |             |             |               |     |
|------------------|--------------------------------------------------------------------------------|-------------|-------------|-------------|---------------|-----|
| [CTNNB1]23<br>41 | mG*mU*mC*mC*mA*A*<br>G*A*T*C*A*G*C*A*G*<br>mU*mC*mU*mC*mA                      | 3.61        | 1.96        | 2.78        | 32.61         | 103 |
| [CTNNB1]24<br>39 | mA*mC*mC*mC*mA*A*<br>G*G*C*A*T*C*C*T*G*m<br>G*mC*mC*mA*mU                      | 4.62        | 2.29        | 3.45        | 70.66         | 104 |
| [CTNNB1]24<br>46 | <b>mG*mG*mU*mC*mC*A</b><br><b>*T*A*C*C*A*A*G*G</b><br><b>*mC*mA*mU*mC*mC</b>   | <b>1.65</b> | <b>1.00</b> | <b>1.33</b> | <b>104.51</b> | 105 |
| [CTNNB1]24<br>47 | mG*mG*mG*mU*mC*C*<br>A*T*A*C*C*A*A*G*<br>mG*mC*mA*mU*mC                        | 1.85        | 1.08        | 1.46        | 47.61         | 106 |
| [CTNNB1]24<br>79 | mG*mG*mU*mG*mG*T*<br>G*G*C*C*A*C*C*A*<br>mU*mC*mU*mC*mA                        | 1.78        | 1.11        | 1.44        | 75.57         | 107 |
| [CTNNB1]25<br>16 | mA*mG*mA*mU*mC*T*<br>G*G*C*A*G*C*C*A*<br>mU*mC*mA*mA*mC                        | 3.15        | 1.89        | 2.52        | 55.37         | 108 |
| [CTNNB1]25<br>45 | mG*mC*mC*mC*mA*T*<br>C*C*A*T*G*A*G*G*T*m<br>C*mC*mU*mG*mG                      | 2.41        | 1.46        | 1.94        | 31.68         | 109 |
| [CTNNB1]27<br>42 | mU*mC*mA*mA*mA*G*<br>T*A*T*A*T*A*C*T*m<br>G*mU*mU*mU*mU                        | 7.84        | 4.27        | 6.06        | 64.39         | 110 |
| [CTNNB1]31<br>1  | mG*mC*mC*mG*mC*T*<br>T*T*T*C*T*G*T*C*T*m<br>G*mG*mU*mU*mC                      | 3.29        | 2.00        | 2.64        | 70.52         | 111 |
| [CTNNB1]33<br>95 | mC*mA*mG*mA*mU*T*<br>A*C*A*A*T*T*A*A*T*m<br>U*mA*mG*mA*mG                      | 4.38        | 2.76        | 3.57        | 80.81         | 112 |
| [CTNNB1]34<br>01 | mU*mU*mU*mA*mU*T*<br>C*A*G*A*T*T*A*C*A*m<br>A*mU*mU*mA*mA                      | 6.77        | 3.68        | 5.23        | 53.38         | 113 |
| [CTNNB1]34<br>45 | <b>mU*mC*mU*mA*mU*T*</b><br><b>T*G*T*C*T*A*T*T*T*</b><br><b>mU*mA*mU*mA*mC</b> | <b>7.84</b> | <b>4.61</b> | <b>6.22</b> | <b>92.76</b>  | 114 |

|                  |                                                           |      |      |      |        |     |
|------------------|-----------------------------------------------------------|------|------|------|--------|-----|
| [CTNNB1]34<br>76 | mC*mA*mU*mA*mU*T*<br>A*A*A*A*A*G*G*A*A*<br>mA*mC*mU*mA*mA | 5.60 | 2.31 | 3.95 | 108.39 | 115 |
| [CTNNB1]34<br>83 | mU*mU*mU*mU*mA*A*<br>G*C*A*T*A*T*T*A*A*m<br>A*mA*mA*mG*mG | 7.17 | 3.67 | 5.42 | 59.36  | 116 |
| [CTNNB1]34<br>84 | mA*mU*mU*mU*mU*A*<br>A*G*C*A*T*A*T*T*A*m<br>A*mA*mA*mA*mG | 5.91 | 2.37 | 4.14 | 47.01  | 117 |
| [CTNNB1]34<br>85 | mU*mA*mU*mU*mU*T*<br>A*A*G*C*A*T*A*T*T*m<br>A*mA*mA*mA*mA | 6.24 | 2.61 | 4.43 | 62.33  | 118 |
| [CTNNB1]34<br>86 | mU*mU*mA*mU*mU*T*<br>T*A*A*G*C*A*T*A*T*m<br>U*mA*mA*mA*mA | 7.17 | 2.97 | 5.07 | 41.46  | 119 |
| [CTNNB1]34<br>87 | mC*mU*mU*mA*mU*T*<br>T*T*A*A*G*C*A*T*A*<br>mU*mU*mA*mA*mA | 6.72 | 2.88 | 4.80 | 123.13 | 120 |
| [CTNNB1]34<br>88 | mG*mC*mU*mU*mA*T*<br>T*T*T*A*A*G*C*A*T*m<br>A*mU*mU*mA*mA | 5.17 | 1.90 | 3.53 | 41.08  | 121 |
| [CTNNB1]34<br>89 | mU*mG*mC*mU*mU*A*<br>T*T*T*T*A*A*G*C*A*m<br>U*mA*mU*mU*mA | 6.87 | 2.73 | 4.80 | 66.36  | 122 |
| [CTNNB1]36<br>48 | mC*mU*mC*mU*mU*G*<br>A*A*G*C*A*T*C*G*T*m<br>A*mU*mC*mA*mC | 5.00 | 2.71 | 3.86 | 93.38  | 123 |
| [CTNNB1]36<br>97 | mC*mG*mA*mA*mU*G*<br>*A*A*T*T*A*A*A*G*<br>mU*mU*mU*mA*mA  | 5.93 | 3.01 | 4.47 | 104.39 | 124 |
| [CTNNB1]57<br>7  | mG*mG*mA*mU*mC*T*<br>G*C*A*T*G*C*C*T*m<br>C*mA*mU*mC*mU   | 2.02 | 1.15 | 1.59 | 47.92  | 125 |
| [CTNNB1]58<br>9  | mA*mC*mU*mG*mU*G*<br>*T*A*G*A*T*G*G*A*<br>*mU*mC*mU*mG*mC | 4.97 | 3.43 | 4.20 | 99.81  | 126 |

|             |                                                           |      |      |      |       |     |
|-------------|-----------------------------------------------------------|------|------|------|-------|-----|
| [CTNNB1]808 | mC*mG*mU*mG*mU*C*<br>T*G*G*A*A*G*C*T*T*m<br>C*mC*mU*mU*mU | 5.09 | 2.89 | 3.99 | 66.99 | 127 |
| [CTNNB1]809 | mG*mC*mG*mU*mG*T*<br>C*T*G*G*A*A*G*C*T*m<br>U*mC*mC*mU*mU | 1.28 | 0.91 | 1.10 | 40.74 | 128 |
| [CTNNB1]811 | mU*mA*mG*mC*mG*T*<br>G*T*C*T*G*G*A*A*G*<br>mC*mU*mU*mC*mC | 2.85 | 1.56 | 2.21 | 60.29 | 129 |
| [CTNNB1]916 | mG*mG*mG*mA*mA*A*<br>G*G*T*T*A*T*G*C*A*m<br>A*mG*mG*mU*mC | 2.82 | 1.76 | 2.29 | 51.43 | 130 |
| [EGFR]1010  | mC*mU*mU*mG*mC*A*<br>C*G*T*G*G*C*T*T*C*m<br>G*mU*mC*mU*mC | 4.70 | 3.06 | 3.88 | 28.14 | 131 |
| [EGFR]1013  | mG*mU*mC*mC*mU*T*<br>G*C*A*C*G*T*G*G*C*<br>mU*mU*mC*mG*mU | 1.59 | 0.80 | 1.19 | 31.52 | 132 |
| [EGFR]1014  | mU*mG*mU*mC*mC*T*<br>T*G*C*A*C*G*T*G*G*m<br>C*mU*mU*mC*mG | 6.13 | 2.61 | 4.37 | 62.73 | 133 |
| [EGFR]1015  | mG*mU*mG*mU*mC*C*<br>T*T*G*C*A*C*G*T*G*m<br>G*mC*mU*mU*mC | 1.55 | 0.80 | 1.17 | 28.29 | 134 |
| [EGFR]1016  | mG*mG*mU*mG*mU*C*<br>C*T*T*G*C*A*C*G*T*m<br>G*mG*mC*mU*mU | 1.58 | 0.88 | 1.23 | 72.16 | 135 |
| [EGFR]1017  | mA*mG*mG*mU*mG*T*<br>C*C*T*T*G*C*A*C*G*m<br>U*mG*mG*mC*mU | 2.42 | 1.27 | 1.84 | 76.42 | 136 |
| [EGFR]1018  | mC*mA*mG*mG*mU*G*<br>T*C*C*T*T*G*C*A*C*m<br>G*mU*mG*mG*mC | 3.41 | 1.95 | 2.68 | 55.43 | 137 |
| [EGFR]1115  | mG*mG*mG*mA*mC*A*<br>C*T*T*C*T*T*C*A*C*m<br>G*mC*mA*mG*mG | 2.13 | 1.24 | 1.69 | 52.15 | 138 |

|            |                                                             |      |      |      |       |     |
|------------|-------------------------------------------------------------|------|------|------|-------|-----|
| [EGFR]1224 | mA*mA*mG*mG*mC*C*<br>C*T*T*C*G*C*A*C*T*m<br>U*mC*mU*mU*mA   | 2.54 | 1.49 | 2.02 | 31.16 | 139 |
| [EGFR]1225 | mC*mA*mA*mG*mG*C*<br>C*C*T*T*C*G*C*A*C*m<br>U*mU*mC*mU*mU   | 4.44 | 2.42 | 3.43 | 79.44 | 140 |
| [EGFR]1226 | mG*mC*mA*mA*mG*G*<br>C*C*C*T*T*C*G*C*A*m<br>C*mU*mU*mC*mU   | 4.33 | 2.55 | 3.44 | 33.01 | 141 |
| [EGFR]2313 | mC*mA*mC*mU*mG*G*<br>G*T*G*T*A*A*G*A*G*<br>mG*mC*mU*mC*mC   | 3.94 | 2.44 | 3.19 | 87.28 | 142 |
| [EGFR]2790 | mC*mU*mG*mU*mG*A*<br>T*C*T*T*G*A*A*C*A*T*m<br>G*mC*mU*mG*mC | 5.24 | 3.44 | 4.34 | 53.32 | 143 |
| [EGFR]2859 | mU*mA*mG*mG*mC*A*<br>C*T*T*T*G*C*C*T*C*m<br>C*mU*mU*mC*mU   | 5.45 | 2.29 | 3.87 | 58.92 | 144 |
| [EGFR]2874 | mA*mU*mG*mC*mC*A*<br>T*C*C*A*C*T*T*G*A*m<br>U*mA*mG*mG*mC   | 2.49 | 1.16 | 1.83 | 29.28 | 145 |
| [EGFR]3263 | mA*mU*mC*mC*mA*C*<br>C*A*C*G*T*C*G*T*C*m<br>C*mA*mU*mG*mU   | 1.79 | 1.06 | 1.42 | 34.71 | 146 |
| [EGFR]3266 | mG*mG*mC*mA*mU*C*<br>C*A*C*C*A*C*G*T*C*m<br>G*mU*mC*mC*mA   | 1.53 | 0.85 | 1.19 | 34.94 | 147 |
| [EGFR]3267 | mC*mG*mG*mC*mA*T*<br>C*C*A*C*C*A*C*G*T*m<br>C*mG*mU*mC*mC   | 2.97 | 1.33 | 2.15 | 56.74 | 148 |
| [EGFR]3268 | mU*mC*mG*mG*mC*A*<br>T*C*C*A*C*C*A*C*G*m<br>U*mC*mG*mU*mC   | 2.37 | 1.39 | 1.88 | 53.73 | 149 |
| [EGFR]3274 | mU*mA*mC*mU*mC*G*<br>T*C*G*G*C*A*T*C*C*m<br>A*mC*mC*mA*mC   | 2.71 | 1.08 | 1.90 | 70.15 | 150 |

|            |                                                           |      |      |      |       |     |
|------------|-----------------------------------------------------------|------|------|------|-------|-----|
| [EGFR]3275 | mG*mU*mA*mC*mU*C*<br>G*T*C*G*G*C*A*T*C*m<br>C*mA*mC*mC*mA | 2.11 | 1.25 | 1.68 | 31.46 | 151 |
| [EGFR]335  | mG*mU*mU*mA*mC*T*<br>C*G*T*G*C*C*T*T*G*m<br>G*mC*mA*mA*mA | 1.93 | 1.17 | 1.55 | 32.34 | 152 |
| [EGFR]3526 | mC*mU*mU*mU*mU*G*<br>G*G*A*A*C*G*G*A*C*<br>mU*mG*mG*mU*mU | 3.17 | 1.91 | 2.54 | 53.24 | 153 |
| [EGFR]3652 | mA*mC*mA*mG*mU*G*<br>T*T*G*A*G*A*T*A*C*m<br>U*mC*mG*mG*mG | 5.02 | 2.73 | 3.88 | 47.65 | 154 |
| [EGFR]3908 | mG*mG*mG*mU*mA*T*<br>C*G*A*A*A*G*A*G*T*<br>mC*mU*mG*mG*mA | 1.35 | 0.91 | 1.13 | 27.08 | 155 |
| [EGFR]4705 | mU*mG*mG*mG*mC*T*<br>G*G*A*A*T*C*C*G*A*<br>mG*mU*mU*mA*mU | 2.93 | 1.92 | 2.42 | 36.11 | 156 |
| [EGFR]4885 | mG*mG*mA*mG*mA*T*<br>T*T*C*A*G*A*G*C*A*m<br>G*mC*mU*mU*mC | 1.87 | 1.25 | 1.56 | 36.25 | 157 |
| [EGFR]5094 | mU*mU*mA*mC*mU*T*<br>T*A*A*A*A*G*C*A*A*<br>mA*mA*mG*mG*mA | 5.74 | 2.27 | 4.00 | 34.97 | 158 |
| [EGFR]5095 | mU*mG*mA*mA*mG*T*<br>A*A*A*A*A*T*C*A*A*<br>mU*mA*mG*mC*mG | 3.99 | 1.96 | 2.97 | 29.97 | 159 |
| [EGFR]5101 | mG*mU*mA*mA*mA*A*<br>A*G*C*T*T*T*T*G*A*<br>A*mG*mU*mG*mA  | 3.82 | 1.62 | 2.72 | 29.11 | 160 |
| [EGFR]5102 | mU*mU*mG*mA*mA*G*<br>T*G*A*A*G*T*A*A*A*<br>mA*mG*mG*mA*mG | 1.31 | 0.96 | 1.13 | 31.83 | 161 |
| [EGFR]5103 | mU*mU*mU*mU*mG*A*<br>A*G*T*G*T*T*A*A*m<br>U*mA*mU*mU*mC   | 7.84 | 4.09 | 5.96 | 78.92 | 162 |

|            |                                                           |      |      |      |       |     |
|------------|-----------------------------------------------------------|------|------|------|-------|-----|
| [EGFR]5104 | mG*mU*mA*mA*mA*A*<br>G*G*A*G*A*A*A*A*C*<br>mU*mA*mU*mC*mU | 3.05 | 1.14 | 2.10 | 31.74 | 163 |
| [EGFR]5105 | mU*mU*mU*mG*mA*A*<br>G*T*G*A*A*G*T*A*A*<br>mA*mA*mG*mG*mA | 5.70 | 2.58 | 4.14 | 35.25 | 164 |
| [EGFR]5106 | mU*mU*mU*mA*mC*G*<br>G*T*T*T*T*C*A*G*A*m<br>A*mU*mA*mU*mC | 7.84 | 3.63 | 5.74 | 40.61 | 165 |
| [EGFR]5107 | mG*mA*mA*mG*mU*G*<br>A*A*G*T*A*A*A*G*<br>mG*mA*mG*mA*mA   | 0.78 | 0.57 | 0.67 | 35.65 | 166 |
| [EGFR]5108 | mA*mA*mA*mA*mG*G*<br>A*G*A*A*A*A*C*T*A*<br>mU*mC*mU*mU*mC | 4.36 | 1.65 | 3.01 | 47.60 | 167 |
| [EGFR]5110 | mA*mA*mA*mA*mA*T*<br>T*A*C*T*T*T*A*A*A*m<br>A*mG*mC*mA*mA | 3.90 | 1.32 | 2.61 | 71.09 | 168 |
| [EGFR]5111 | mA*mG*mU*mA*mA*A*<br>A*A*G*C*T*T*T*G*m<br>A*mA*mG*mU*mG   | 5.34 | 2.70 | 4.02 | 48.30 | 169 |
| [EGFR]5112 | mU*mU*mU*mU*mG*A*<br>A*G*T*G*A*A*G*T*A*<br>mA*mA*mA*mG*mG | 6.17 | 3.08 | 4.62 | 51.65 | 170 |
| [EGFR]5113 | mG*mU*mA*mG*mA*G*<br>A*A*A*T*T*A*T*T*T*m<br>U*mA*mG*mG*mA | 4.92 | 2.47 | 3.70 | 34.45 | 171 |
| [EGFR]5114 | mA*mA*mU*mU*mA*C*<br>T*T*T*A*A*A*A*G*C*m<br>A*mA*mA*mA*mG | 5.13 | 2.07 | 3.60 | 77.10 | 172 |
| [EGFR]5115 | mU*mG*mU*mA*mG*A*<br>G*A*A*A*T*T*A*T*T*m<br>U*mU*mA*mG*mG | 6.58 | 2.99 | 4.78 | 61.44 | 173 |
| [EGFR]5118 | mA*mU*mU*mA*mC*T*<br>T*T*A*A*A*A*G*C*A*<br>mA*mA*mA*mG*mG | 5.43 | 2.17 | 3.80 | 42.87 | 174 |

|                    |                                                                                |              |             |              |              |            |
|--------------------|--------------------------------------------------------------------------------|--------------|-------------|--------------|--------------|------------|
| [EGFR]5119         | mU*mU*mU*mU*mU*G*<br>A*A*G*T*G*T*T*A*m<br>A*mU*mA*mU*mU                        | 7.84         | 3.43        | 5.63         | 59.02        | 175        |
| [EGFR]5120         | mU*mA*mA*mA*mA*G*<br>G*A*G*A*A*A*A*C*T*<br>mA*mU*mC*mU*mU                      | 3.93         | 1.69        | 2.81         | 58.22        | 176        |
| [EGFR]5121         | mC*mU*mU*mU*mU*G*<br>A*A*G*T*G*A*A*G*T*<br>mA*mA*mA*mA*mG                      | 3.76         | 1.98        | 2.87         | 79.85        | 177        |
| [EGFR]727          | mA*mU*mG*mU*mC*C*<br>C*G*C*C*A*C*T*G*G*m<br>A*mU*mG*mC*mU                      | 1.58         | 0.84        | 1.21         | 71.68        | 178        |
| [LINC-<br>PINT]101 | mU*mC*mC*mC*mA*T*<br>C*C*C*T*T*C*T*G*C*m<br>U*mG*mC*mC*mA                      | 10.88        | 3.95        | 17.01        | 43.08        | 179        |
| [LINC-<br>PINT]102 | mG*mU*mC*mC*mC*A*<br>T*C*C*C*T*T*C*T*G*m<br>C*mU*mG*mC*mC                      | 5.07         | 1.37        | 17.73        | 22.47        | 180        |
| [LINC-<br>PINT]103 | mG*mG*mU*mC*mC*C*<br>A*T*C*C*C*T*T*C*T*m<br>G*mC*mU*mG*mC                      | 1.87         | 1.28        | 16.85        | 20.75        | 181        |
| [LINC-<br>PINT]106 | mU*mC*mU*mG*mG*T*<br>C*C*C*A*T*C*C*C*T*m<br>U*mC*mU*mG*mC                      | 5.25         | 3.36        | 16.79        | 29.42        | 182        |
| [LINC-<br>PINT]108 | mU*mC*mU*mC*mU*G*<br>G*T*C*C*C*A*T*C*C*m<br>C*mU*mU*mC*mU                      | <u>11.42</u> | <u>5.44</u> | <u>33.24</u> | <u>78.25</u> | <u>183</u> |
| [LINC-<br>PINT]109 | <u>mC*mU*mC*mU*mC*T*</u><br><u>G*G*T*C*C*C*A*T*C*</u><br><u>mC*mC*mU*mU*mC</u> | <u>10.10</u> | <u>2.73</u> | <u>40.55</u> | <u>84.97</u> | <u>184</u> |
| [LINC-<br>PINT]110 | <u>mU*mC*mU*mC*mU*C*</u><br><u>T*G*G*T*C*C*C*A*T*m</u><br><u>C*mC*mC*mU*mU</u> | <u>11.27</u> | <u>3.68</u> | <u>20.35</u> | <u>43.82</u> | <u>185</u> |
| [LINC-<br>PINT]111 | <u>mU*mU*mC*mU*mC*T*</u><br><u>C*T*G*G*T*C*C*C*A*m</u><br><u>U*mC*mC*mC*mU</u> | <u>9.51</u>  | <u>2.83</u> | <u>17.22</u> | <u>26.22</u> | <u>186</u> |

|                      |                                                                                |              |             |              |              |            |
|----------------------|--------------------------------------------------------------------------------|--------------|-------------|--------------|--------------|------------|
| [LINC-<br>PINT]112   | <u>mC*mU*mU*mC*mU*C*</u><br><u>T*C*T*G*T*C*C*m</u><br><u>A*mU*mC*mC*mC</u>     | <u>9.21</u>  | <u>2.92</u> | <u>22.69</u> | <u>56.20</u> | <u>187</u> |
| [LINC-<br>PINT]113   | <u>mC*mC*mU*mU*mC*T*</u><br><u>C*T*C*T*G*G*T*C*m</u><br><u>C*mA*mU*mC*mC</u>   | <u>3.51</u>  | <u>3.01</u> | <u>23.33</u> | <u>60.90</u> | <u>188</u> |
| [LINC-<br>PINT]114   | <u>mC*mC*mC*mU*mU*C*</u><br><u>T*C*T*C*T*G*G*T*C*m</u><br><u>C*mC*mA*mU*mC</u> | <u>8.12</u>  | <u>2.60</u> | <u>18.76</u> | <u>49.59</u> | <u>189</u> |
| [LINC-<br>PINT]115   | <u>mA*mC*mC*mC*mU*T*</u><br><u>C*T*C*T*C*T*G*G*T*m</u><br><u>C*mC*mC*mA*mU</u> | <u>7.57</u>  | <u>2.55</u> | <u>18.30</u> | <u>43.37</u> | <u>190</u> |
| [LINC-<br>PINT]116   | <u>mC*mA*mC*mC*mC*T*</u><br><u>T*C*T*C*T*C*T*G*G*m</u><br><u>U*mC*mC*mC*mA</u> | <u>5.97</u>  | <u>1.85</u> | <u>27.63</u> | <u>73.30</u> | <u>191</u> |
| [LINC-<br>PINT]1222  | <u>mU*mU*mA*mG*mC*T*</u><br><u>C*C*T*T*G*C*C*T*C*m</u><br><u>G*mU*mU*mC*mC</u> | <u>8.41</u>  | <u>3.43</u> | <u>19.40</u> | <u>31.67</u> | <u>192</u> |
| [LINC-<br>PINT]126   | <u>mG*mU*mC*mU*mC*C*</u><br><u>T*C*C*A*C*A*C*C*m</u><br><u>U*mU*mC*mU*mC</u>   | <u>7.17</u>  | <u>1.98</u> | <u>18.29</u> | <u>25.01</u> | <u>193</u> |
| [LINC-<br>PINT]127   | <u>mG*mG*mU*mC*mU*C*</u><br><u>C*T*C*C*A*C*A*C*m</u><br><u>C*mU*mU*mC*mU</u>   | <u>3.72</u>  | <u>1.29</u> | <u>19.32</u> | <u>23.66</u> | <u>194</u> |
| [LINC-<br>PINT]128   | <u>mG*mG*mG*mU*mC*T*</u><br><u>C*C*T*C*C*A*C*A*m</u><br><u>C*mC*mU*mU*mC</u>   | <u>1.23</u>  | <u>0.87</u> | <u>18.20</u> | <u>21.47</u> | <u>195</u> |
| [LINC-<br>PINT]1284  | <u>mU*mC*mC*mC*mA*A*</u><br><u>C*T*C*T*T*C*T*A*A*m</u><br><u>C*mU*mC*mG*mU</u> | <u>13.35</u> | <u>3.70</u> | <u>19.85</u> | <u>40.79</u> | <u>196</u> |
| [LINC-<br>PINT]1315  | <u>mG*mC*mA*mA*mG*G*</u><br><u>C*A*G*A*G*A*A*A*C*</u><br><u>mU*mC*mC*mA*mG</u> | <u>2.69</u>  | <u>1.45</u> | <u>17.16</u> | <u>19.99</u> | <u>197</u> |
| [LINC-<br>PINT]148.1 | <u>mA*mA*mA*mU*mG*T*</u><br><u>C*C*T*G*G*C*C*T*m</u><br><u>C*mA*mC*mU*mG</u>   | <u>1.56</u>  | <u>1.45</u> | <u>15.38</u> | <u>46.45</u> | <u>198</u> |

|                       |                                                           |       |      |       |        |     |
|-----------------------|-----------------------------------------------------------|-------|------|-------|--------|-----|
| [LINC-<br>PINT]1497.1 | mG*mA*mU*mG*mG*T*<br>T*C*C*A*G*T*C*C*m<br>U*mC*mU*mU*mC   | 1.01  | 0.84 | 15.35 | 22.32  | 199 |
| [LINC-<br>PINT]2504   | mC*mC*mU*mA*mU*T*<br>A*A*A*A*A*A*T*T*<br>mU*mA*mU*mA*mC   | 6.09  | 2.19 | 76.27 | 108.68 | 200 |
| [LINC-<br>PINT]2524   | mU*mA*mU*mU*mC*A<br>*T*A*T*T*T*T*A*T*<br>mU*mU*mC*mA*mG   | 10.13 | 4.47 | 45.95 | 87.12  | 201 |
| [LINC-<br>PINT]2527   | mU*mG*mC*mU*mA*T*<br>T*C*A*T*A*T*T*T*m<br>U*mA*mU*mU*mU   | 9.02  | 3.01 | 18.42 | 42.38  | 202 |
| [LINC-<br>PINT]2673.1 | mU*mU*mG*mG*mC*C*<br>T*G*T*G*G*A*T*G*C*m<br>U*mU*mU*mG*mU | 0.94  | 0.75 | 13.85 | 21.43  | 203 |
| [LINC-<br>PINT]2690   | mU*mU*mU*mG*mA*A*<br>A*T*T*C*A*G*A*A*G*<br>mA*mU*mU*mU*mG | 4.93  | 2.57 | 20.33 | 35.07  | 204 |
| [LINC-<br>PINT]2754   | mU*mU*mU*mA*mU*A*<br>T*T*A*C*A*A*A*G*C*m<br>U*mA*mC*mU*mU | 6.45  | 2.71 | 16.54 | 33.70  | 205 |
| [LINC-<br>PINT]2755   | mC*mU*mU*mU*mA*T*<br>A*T*T*A*C*A*A*A*G*<br>mC*mU*mA*mC*mU | 6.37  | 2.11 | 22.54 | 54.83  | 206 |
| [LINC-<br>PINT]2811   | mA*mA*mA*mA*mG*T*<br>G*G*G*A*A*A*T*A*A*<br>mA*mG*mG*mU*mU | 2.33  | 1.48 | 14.66 | 30.90  | 207 |
| [LINC-<br>PINT]2812   | mA*mA*mA*mA*mA*G*<br>T*G*G*G*A*A*A*T*A*<br>mA*mA*mG*mG*mU | 1.83  | 1.34 | 15.63 | 28.76  | 208 |
| [LINC-<br>PINT]283.1  | mU*mG*mA*mU*mG*A*<br>T*G*C*T*T*G*C*A*G*m<br>G*mA*mG*mG*mC | 1.57  | 1.18 | 16.66 | 25.50  | 209 |
| [LINC-<br>PINT]2990   | mC*mA*mC*mU*mG*T*<br>A*T*T*T*A*T*T*A*m<br>C*mA*mG*mA*mA   | 4.86  | 2.67 | 18.87 | 58.13  | 210 |

|                      |                                                           |      |      |       |       |     |
|----------------------|-----------------------------------------------------------|------|------|-------|-------|-----|
| [LINC-<br>PINT]3011  | mA*mG*mU*mU*mU*A*<br>T*A*G*A*T*T*T*C*A*m<br>A*mG*mU*mA*mG | 5.28 | 3.06 | 17.44 | 37.64 | 211 |
| [LINC-<br>PINT]384   | mU*mG*mA*mC*mA*A*<br>A*A*C*A*A*T*A*A*T*<br>mA*mA*mC*mA*mG | 2.57 | 1.31 | 18.59 | 36.68 | 212 |
| [LINC-<br>PINT]412   | mG*mU*mU*mC*mA*G*<br>T*C*A*G*A*T*C*G*C*m<br>U*mG*mG*mG*mA | 1.70 | 1.55 | 16.42 | 17.46 | 213 |
| [LINC-<br>PINT]450   | mA*mA*mA*mG*mU*C*<br>A*A*A*A*A*G*A*A*A*<br>mA*mA*mC*mU*mG | 2.72 | 1.62 | 18.78 | 45.57 | 214 |
| [LINC-<br>PINT]501   | mU*mG*mU*mU*mU*C*<br>C*C*C*G*G*A*G*A*G*<br>mC*mA*mA*mU*mG | 2.04 | 1.62 | 15.46 | 30.90 | 215 |
| [LINC-<br>PINT]523   | mU*mG*mA*mC*mA*T*<br>T*T*C*G*T*G*G*C*T*m<br>C*mC*mU*mA*mC | 4.34 | 1.73 | 15.98 | 33.56 | 216 |
| [LINC-<br>PINT]524.1 | mA*mU*mG*mA*mC*A*<br>T*T*T*C*G*T*G*G*C*m<br>U*mC*mC*mU*mA | 1.20 | 0.88 | 17.18 | 32.53 | 217 |
| [LINC-<br>PINT]587   | mA*mG*mC*mC*mG*A*<br>A*C*A*G*A*A*G*G*A*<br>mG*mC*mG*mU*mC | 3.73 | 2.54 | 17.17 | 27.70 | 218 |
| [LINC-<br>PINT]727.1 | mG*mU*mC*mC*mG*T*<br>A*C*C*T*C*C*A*C*C*m<br>C*mA*mC*mC*mG | 3.70 | 1.70 | 15.34 | 22.79 | 219 |
| [LINC-<br>PINT]83.1  | mC*mA*mA*mG*mC*C*<br>C*C*A*G*C*G*T*T*C*m<br>C*mU*mC*mC*mG | 4.44 | 2.74 | 23.97 | 45.20 | 220 |
| [LINC-<br>PINT]877   | mC*mC*mC*mU*mA*A*<br>T*G*C*T*T*T*C*C*T*m<br>C*mU*mC*mC*mA | 8.39 | 2.70 | 32.41 | 58.39 | 221 |
| [LINC-<br>PINT]935   | mG*mC*mG*mU*mA*G*<br>T*T*T*C*T*C*T*T*C*m<br>C*mU*mC*mC*mC | 4.12 | 1.86 | 18.79 | 27.23 | 222 |

|                  |                                                                                |             |             |              |              |     |
|------------------|--------------------------------------------------------------------------------|-------------|-------------|--------------|--------------|-----|
| [LINC-PINT]937.1 | mU*mG*mG*mC*mG*T*<br>A*G*T*T*T*C*T*C*T*m<br>U*mC*mC*mU*mC                      | 4.25        | 2.48        | 15.25        | 32.81        | 223 |
| [LINC-PINT]94    | <b>mC*mC*mU*mU*mC*T*</b><br><b>G*C*T*G*C*C*A*A*G*</b><br><b>mC*mC*mC*mC*mA</b> | <b>3.71</b> | <b>1.45</b> | <b>33.74</b> | <b>85.57</b> | 224 |
| [LINC-PINT]98    | mC*mA*mU*mC*mC*C*<br>T*T*C*T*G*C*T*G*C*m<br>C*mA*mA*mG*mC                      | 8.23        | 2.95        | 24.38        | 54.34        | 225 |
| [LINC-PINT]99    | mC*mC*mA*mU*mC*C*<br>C*T*T*C*T*G*C*T*G*m<br>C*mC*mA*mA*mG                      | 5.67        | 2.38        | 17.14        | 52.57        | 226 |
|                  | R848 only                                                                      | 1.00        | 1.00        | 100.00       | 100.00       |     |
|                  | NT                                                                             | 0.20        | 0.07        | 13.17        | 26.54        |     |

**EXAMPLE 4: TLR7 inhibition by 2'OMe ASOs can be reverted by CUU terminal motifs**

MEME motif discovery analysis (Bailey and Elkan, 1994) of 19 ASOs with lowest TLR7 inhibitory activity based on the above screens led to the observation that the 2'OMe regions of the ASOs exhibiting terminal 5' and 3' "C" bases were over-represented in 8 sequences, along with uridine residues (Figure 3A, Table 2). In addition, analysis of a family of sequences from the screen (referred to as the PINT family) with single base increments suggested different inhibitory activity on TLR7 for closely related sequences (Figure 3B, 3C and Table 2 - from ASO '[LINC-PINT]-108' to [LINC-PINT]-116', referred to as ASO108-ASO116).

Validation of the PINT family of ASOs in HEK293-TLR7 cells confirmed that ASO111 only was capable of blocking TLR7 activation by R848 in this family (Figures 3B, 3C). Critically, sequence alignment analyses revealed that ASO111 was the only sequence lacking a CUU/CUT/CTT motif in its 5' or 3' end regions (Figure 3C). Since [cGAS]ASO11 also harboured such terminal 2'OMe CUU motifs in both its 5' and 3'-ends (along with 4 other sequences harbouring the enriched motif in Figure 3A), the inventors next tested [cGAS]ASO11 variants (ASO11-Mut1 and ASO11-Mut2) in which the 5' and 3' end 2'OMe regions were swapped with these of ASO2, which lacked such "CUU" motifs and was a potent repressor of TLR7 (Figures 3D, 3E). Aligning with a

key role for both the 5' and 3' end regions, both ASO11 mutants significantly increased TLR7 inhibition compared to the parental ASO11 (Figures 3D, 3E). These findings collectively indicated that 5' and 3' CUU motifs were important modulators of TLR7 inhibition by the 2'OMe-PS ASOs.

5

**EXAMPLE 5: TLR8 potentiation is driven by the central DNA region and the 5' end of 2'OMe PS ASOs**

The inventors also analysed the PINT family (ASO108-116) and [cGAS]ASO11 mutants for TLR8 potentiation. While two sequences were more potent (e.g. ASO108 and ASO110), most of the sequences displayed similar TLR8 potentiation, suggesting that the central region of these molecules was predominantly involved in TLR8 modulation (Figures 3C, 3F). Similarly, the ASO11 mutations only mildly impacted TLR8 potentiation, although addition of the ASO2 3' end (ASO11-Mut1) significantly decreased, while the ASO2 5' end (ASO11-Mut2) significantly increased TLR8 sensing (Figures 3D, 3G). These results indicated that the control of TLR8 potentiation by 2'OMe-PS ASO was predominantly governed by the central 10-mer DNA region of the ASO, but that the 2'OMe ends also played a role.

In further support of a contribution for the 5'-end region of the ASOs, the inventors noticed that most of the top 20 potentiators of TLR8 sensing in our screen had a terminal 5'-U (14 out of 20), while occurrence of such terminal 5'-U was much less frequent among the bottom 20 potentiators (3 out of 20) (Table 2). Analyses of TLR8 potentiation on the 192 ASOs comparing sequences with or without terminal 5'-U confirmed a significantly increased potentiation of TLR8 sensing for sequences harbouring a terminal 5'-U (Figure 3H and Table 2). A similar trend was observed considering terminal 5'-UC motifs (present in 9/20 of the top ASOs - Figure 3I and Table 2). Critically, ASO108 and ASO110 were the only two ASOs from the PINT family to harbour such terminal 5'-UC motif, also present in ASO2 and ASO11-Mut2.

The inventors also noted that the central 10-mer DNA region of the TLR8 potentiating ASO852 contained a central T-rich region (TTTCTGTGGT), while that of ASO2504 was A-rich (TAAAAAAATT). Comparison of the central DNA regions of the top and bottom 20 potentiators of TLR8 sensing confirmed a significant increased proportion of thymidine residues in the ASOs potentiating TLR8 sensing the most – with a median of 4 central thymidines (Figure 3J and Table 2). Since this was directly aligned with previous reports that T-rich regions were important for TLR8 potentiation (Gorden et al., 2006; Jurk et al., 2006), the inventors mutated ASO852 to ASO852-dT, containing a central stretch of 10 dTs (Figure 3K). In addition, the inventors swapped the central 10-

mer DNA region of ASO2504 with that of ASO852 to result in ASO2504-Mut (Figure 3K), since ASO2504 did not potentiate TLR8 as much as ASO852. Comparison of the activities of these oligonucleotides on R848 sensing was performed in THP-1 cells, HEK-TLR7 and HEK-TLR8 cells (Figures 3L, 3M, 8A, 8B and 8D). The 2504-Mut ASO was significantly more potent in driving IP-10 production in THP-1 cells and NF- $\kappa$ B luciferase in HEK-TLR8 cells, an observation in support of a critical role for the central T-rich 10-mer DNA region of the ASOs in their effects on TLR8. In addition, ASO852-dT was also more potent at inducing IP-10 than ASO852, reaching similar levels of stimulation to those obtained with a 20-mer dT PS oligonucleotide (dT20) in THP-1 cells. Conversely, substitutions of the central regions for ASO852-dT and ASO2504-Mut did not significantly impact TLR7 sensing of R848, while dT20 blocked TLR7 activation. None of these ASOs used alone in HEK-TLR7, HEK-TLR8 or THP-1 cells impacted NF- $\kappa$ B luciferase in HEK cells and IP-10 production in THP-1 (Figures 8A, 8B, 8D). However, increasing the central T-rich region of ASO852 potentiated further its basal activity on TLR9-driven NF- $\kappa$ B luciferase – aligning with the observation that dT20 alone also acted as a mild TLR9 ligand in HEK-TLR9 cells (Figure 8C).

#### **EXAMPLE 6: TLR8 potentiation of R848 by ASOs leads to IRF activation**

The capacity of ASO852-dT to strongly potentiate IP-10 production upon R848 sensing was confirmed in two other TLR8 expressing AML cell lines (MOLM13 and OCI-AML3; Figure 4A), and was readily observable with as little as 4 to 20 nM ASO852-dT (Figure 4B). Given the high production of IP-10 seen, suggestive of IRF activation, the inventors tested the activity of the ASO852-dT series in HEKTLR8 cells expressing CCL5-Luciferase or IFN- $\beta$ -Luciferase reporters, which are driven by IRFs (Chow et al., 2018; Schafer et al., 1998). While R848 alone did not activate either reporters, co-stimulation with the oligonucleotides potentiated both promoters, with ASO852-dT and dT20 being the most potent, followed by ASO852/2504-Mut, and ASO2504 being the least potent (Figure 4C); this finding thus mirrors the results obtained with IP-10 production in THP-1 cells (Figure 3L).

To further support induction of an IRF-driven response, the inventors carried out RT-qPCR analyses of several IRF-driven genes including IFNB1, at 4 h after R848 stimulation of THP-1 cells. While little induction of IFIT1, RSAD2, IFI44 and IFNB1 was seen with R848 only, all these genes were significantly increased by co-stimulation with ASO852- dT (Figure 4D). The inventors had observed that ASO co-stimulation strongly increased the sensitivity of TLR8 to R848 in HEK-TLR8 cells (Figure 2D), suggesting that the effect seen on IFN- $\beta$  induction may be due to increased sensitivity of

TLR8 to R848. IFN- $\beta$ -Luciferase reporter dose-responses to R848 (ranging from 1 to 15  $\mu$ g/ml) in HEK-TLR8 cells demonstrated that high doses of R848 engaged the IFN- $\beta$  response in these cells to a similar extent as with low dose R848+ASO852-dT.

Collectively these results suggested that ASO such as ASO852-dT facilitated  
5 activation of IRF-driven response otherwise only achievable with very high doses of R848.

#### **EXAMPLE 7: Identification of gene-targeting ASOs potentiating TLR8 sensing**

The inventors next sought to establish proof-of-principle that bi-functional ASOs  
10 combining gene-targeting and TLR8 potentiation (while avoiding TLR7 inhibition) could be achieved. For this purpose, the inventors tested a panel of 48 2'OMe ASOs designed against the mRNA of the human HPRT gene.

Preliminary studies in HeLa cells suggested that 29 out of the 48 ASOs significantly reduced HPRT mRNA levels by at least 50% at 10 nM (Figure 6 and Table  
15 3). The inventors therefore focused on this sub-panel of 29 sequences for the following experiments comparing gene-targeting, TLR7 inhibition and TLR8 potentiation of R848 sensing (Figure 5A, 5B and 5C, respectively). In agreement with the previous findings, these experiments confirmed that most ASOs blocked TLR7 activation by R848, with the notable exceptions of [HPRT]ASO551, ASO660-663 and ASO665 ASOs (Figure  
20 5B). Critically, each of these sequences harboured at least one CUU/CUT motifs in their 5' or 3' end, further suggesting an important role for these motifs in the retention of TLR7 sensing (Figure 5D). Interestingly, both ASO551 and ASO662 ASOs harboured one 5'-CUU and one 3'-UUC motif, but ASO551 ASO was the only ASO entirely preserving TLR7 sensing. Since the position of these 5'-CUU and 3'-UUC motifs varied  
25 between both ASOs, optimal positioning of the CUU motif may be in the terminal 5'-end of the ASOs, indicating that terminal 3'-end UUC motifs may also be important. At this point, the inventors note again that [LINC-PINT]ASO109 and [cGAS]ASO8 also had such terminal 3'-UUC motifs. These data align with the MEME motif that showed terminal 5' and 3' Cs were prevalent in non-inhibitory sequences (Figure 3A).

**Table 3:** HPRT-targeting ASO sequences. ASOs were synthesised with the following modifications: UPPERCASE alone for DNA, 'm' indicates 2'OMe base modifications, and \* denotes the phosphorothioate backbone. The 29 sequences used in Figure 5 are in bold.

5

| [Transcript Name] and Reference | Sequence                                                 | SEQ ID NO. |
|---------------------------------|----------------------------------------------------------|------------|
| [HPRT]1027                      | <b>mA*mA*mU*mC*mC*G*C*C*A*A*A*G*G*G*mA*mA*mC*mU*mG</b>   | 227        |
| [HPRT]1353                      | <b>mG*mC*mU*mG*mA*C*A*A*A*G*A*T*T*C*A*mC*mU*mG*mG*mU</b> | 228        |
| [HPRT]292                       | <b>mA*mC*mG*mU*mU*C*A*G*T*C*T*G*T*C*mC*mA*mU*mA*mA</b>   | 229        |
| [HPRT]294                       | <b>mA*mG*mA*mC*mG*T*T*C*A*G*T*C*T*G*mU*mC*mC*mA*mU</b>   | 230        |
| [HPRT]326                       | <b>mG*mG*mC*mC*mU*C*C*A*T*C*T*C*T*mU*mC*mA*mU*mC</b>     | 231        |
| [HPRT]329                       | <b>mG*mA*mU*mG*mG*C*C*T*C*C*A*T*C*T*mC*mC*mU*mU*mC</b>   | 232        |
| [HPRT]330                       | <b>mU*mG*mA*mU*mG*G*C*C*T*C*C*A*T*C*mU*mC*mC*mU*mU</b>   | 233        |
| [HPRT]331                       | <b>mG*mU*mG*mA*mU*G*G*C*C*T*C*C*A*T*mC*mU*mC*mC*mU</b>   | 234        |
| [HPRT]332                       | <b>mU*mG*mU*mG*mA*T*G*G*C*C*T*C*C*A*mU*mC*mU*mC*mC</b>   | 235        |
| [HPRT]333                       | <b>mA*mU*mG*mU*mG*A*T*G*G*C*C*T*C*C*A*mU*mC*mU*mC</b>    | 236        |
| [HPRT]334                       | <b>mA*mA*mU*mG*mU*G*A*T*G*G*C*C*T*C*C*mA*mU*mC*mC</b>    | 237        |
| [HPRT]335                       | <b>mC*mA*mA*mU*mG*T*G*A*T*G*G*C*C*T*C*mC*mC*mA*mU*mC</b> | 238        |
| [HPRT]388                       | <b>mA*mU*mC*mC*mA*G*C*A*G*G*T*C*A*G*C*mA*mA*mA*mG*mA</b> | 239        |

|            |                                                       |     |
|------------|-------------------------------------------------------|-----|
| [HPRT]475  | mC*mU*mG*mG*mU*C*A*T*T*A*C*A*A*T*A*mG*mC*mU*mC*m<br>U | 240 |
| [HPRT]489  | mA*mU*mG*mU*mC*C*C*T*G*T*G*A*C*mU*mG*mG*mU*m<br>C     | 241 |
| [HPRT]551  | mC*mU*mU*mC*mC*A*C*A*A*T*C*A*A*G*A*mC*mA*mU*mU*m<br>C | 242 |
| [HPRT]660  | mC*mU*mU*mC*mG*T*G*G*G*T*C*T*T*mU*mU*mC*mA*m<br>C     | 243 |
| [HPRT]661  | mA*mC*mU*mU*mC*G*T*G*G*G*T*C*T*mU*mU*mU*mC*m<br>A     | 244 |
| [HPRT]662  | mC*mA*mC*mU*mU*C*G*T*G*G*G*T*C*mU*mU*mU*mU*<br>mC     | 245 |
| [HPRT]663  | mA*mC*mA*mC*mU*T*C*G*T*G*G*G*T*C*mC*mU*mU*mU*m<br>U   | 246 |
| [HPRT]664  | mA*mA*mC*mA*mC*T*T*C*G*T*G*G*G*T*mC*mC*mU*mU*m<br>U   | 247 |
| [HPRT]665  | mC*mA*mA*mC*mA*C*T*T*C*G*T*G*G*G*mU*mC*mC*mU*m<br>U   | 248 |
| [HPRT]666  | mC*mC*mA*mA*mC*A*C*T*T*C*G*T*G*G*G*mG*mU*mC*mC*m<br>U | 249 |
| [HPRT]667  | mU*mC*mC*mA*mA*C*A*C*T*T*C*G*T*G*G*mG*mG*mU*mC*m<br>C | 250 |
| [HPRT]668  | mA*mU*mC*mC*mA*A*C*A*C*T*T*C*G*T*G*mG*mG*mG*mU*m<br>C | 251 |
| [HPRT]669  | mU*mA*mU*mC*mC*A*A*C*A*C*T*T*C*G*T*mG*mG*mG*mG*m<br>U | 252 |
| [HPRT]692  | mC*mA*mA*mA*mU*C*C*A*A*C*A*A*A*G*T*mC*mU*mG*mG*m<br>C | 253 |
| [HPRT]732  | mU*mA*mG*mU*mC*A*A*G*G*G*C*A*T*A*T*mC*mC*mU*mA*m<br>C | 254 |
| [HPRT]847  | mA*mU*mA*mG*mG*A*C*T*C*A*G*A*T*G*mU*mU*mU*mC*m<br>C   | 255 |
| [HPRT]950  | mC*mU*mA*mA*mA*G*T*A*C*A*A*A*A*C*A*mG*mA*mU*mA*mA     | 256 |
| [HPRT]1292 | mA*mA*mC*mA*mC*T*A*C*T*A*A*A*A*T*A*mA*mU*mU*mC*mC     | 257 |
| [HPRT]1335 | mG*mU*mA*mA*mU*A*A*T*T*G*A*A*C*A*mA*mG*mU*mU*mG       | 258 |

|            |                                                   |     |
|------------|---------------------------------------------------|-----|
| [HPRT]1126 | mC*mU*mA*mA*mU*A*T*A*T*C*T*T*C*T*C*mU*mU*mU*mA*mU | 259 |
| [HPRT]1123 | mA*mU*mA*mU*mA*T*C*T*T*C*T*C*T*T*T*mA*mU*mU*mU*mC | 260 |
| [HPRT]225  | mU*mU*mA*mG*mG*T*A*T*G*C*A*A*A*T*mA*mA*mA*mU*mC   | 261 |
| [HPRT]1121 | mA*mU*mA*mU*mC*T*T*C*T*T*C*T*T*A*T*mU*mU*mC*mU*mU | 262 |
| [HPRT]1194 | mU*mA*mA*mU*mU*A*A*C*A*A*T*A*T*T*C*mA*mA*mU*mC*mA | 263 |
| [HPRT]939  | mA*mA*mC*mA*mG*A*T*A*A*A*A*T*T*C*T*mU*mA*mG*mA*mA | 264 |
| [HPRT]944  | mU*mA*mC*mA*mA*A*A*C*A*G*A*T*A*A*A*mU*mU*mC*mU    | 265 |
| [HPRT]1244 | mU*mC*mU*mU*mU*G*A*T*G*T*G*A*A*A*mU*mU*mG*mA*mC   | 266 |
| [HPRT]1133 | mU*mA*mA*mA*mA*A*A*C*T*A*A*T*A*T*A*mU*mC*mU*mU*mC | 267 |
| [HPRT]938  | mA*mC*mA*mG*mA*T*A*A*A*A*T*T*C*T*T*mA*mG*mA*mA*mG | 268 |
| [HPRT]934  | mA*mU*mA*mA*mA*A*T*T*C*T*T*A*G*A*A*mG*mA*mU*mA*mC | 269 |
| [HPRT]1135 | mA*mU*mU*mA*mA*A*A*A*A*C*T*A*A*T*A*mU*mA*mU*mC*mU | 270 |
| [HPRT]1124 | mA*mA*mU*mA*mU*A*T*C*T*T*C*T*C*T*T*mU*mA*mU*mU*mU | 271 |
| [HPRT]1134 | mU*mU*mA*mA*mA*A*A*A*C*T*A*A*T*A*T*mA*mU*mC*mU*mU | 272 |
| [HPRT]1195 | mA*mU*mA*mA*mU*T*A*A*C*A*A*T*A*T*T*mC*mA*mA*mU*mC | 273 |

In addition, most ASOs significantly potentiated TLR8 sensing to varying degrees, with the exception of 4 HPRT ASOs (ASO329, ASO321, ASO333, ASO666) (Figure 5C). Based on these analyses, the inventors selected ASO662 as an ASO with good HPRT targeting (>70% at 10 nM), retaining TLR7 activity (~80%), and potentiating TLR8 sensing of R848 ~5 fold. In addition, the inventors selected ASO847 as an ASO with high gene targeting activity (>93%), strong TLR7 inhibition and TLR8 potentiating activity close to that of ASO662. The two ASOs were transfected in THP-1 cells and led to significant HPRT downregulation, which was more pronounced for ASO847 - aligning with the data from HeLa cells (Figure 5A and 5E). In addition, following the same transfection protocol, ASO662 strongly potentiated IP-10 production induced by R848 (to a similar level as with the control dT20 oligonucleotide) (Figure 5F). Unexpectedly, ASO847 failed to increase IP-10 production following R848 co-stimulation, which may be attributed to its inhibitory effect on TLR7, which is also functional in THP-1 cells (Gantier et al., 2008).

#### **EXAMPLE 8: TLR8 potentiation – modulation by 5' end motifs**

It has been previously observed that 2'OMe ASOs could potentiate TLR8 sensing of R848 through the sensing of the central 10 DNA bases, and that "T" rich regions were

better potentiators (as seen with ASO-852 and its variant ASO-852dT) (Fig. 3 - Alharbi et al., 2020).

In addition, the inventors showed that changing the 5'end 2'OMe region of ASO11, to include the 5'UCCGG region of ASO2, led to a significant increase in TLR8  
5 potentiation (Fig. 3 - Alharbi et al., 2020). Comparison of TLR8 potentiation of ASOs terminating with a 5'U or 5'UC motif indicated, based on 192 ASOs, that 5'U or 5'UC ASOs were significantly stronger TLR8 potentiators (Alharbi et al., 2020).

To directly implicate the role of 5'U/UC in the modulation of TLR8 sensing, the inventors next tested the effect of adding a terminal 2'OMe UC motif to an otherwise  
10 non-TLR8 potentiating ASOs. The first molecule the inventors tested was ASO1-UC, which is a 22 nt molecule with appended 5'UC motif (with 7 2'OMe bases on the 5' end). While ASO1 did not potentiate TLR8 in HEKTLR8 or THP-1 cells, its 5'end variant significantly promoted TLR8 potentiation in both models (Figure 9). This is proof of principle that addition of a 5' end UC motif could be used as a strategy to confer TLR8  
15 potentiation to an otherwise non-potentiating 2'OMe gapmer ASOs.

The inventors also tested the effect of 5'end modification of ASO HPRT-660, which the inventors previously found was a strong potentiator of TLR8 (Fig. 5 - Alharbi et al., 2020). The native sequence of ASO 660 contains a 5'CUU region, which the inventors mutated to a 5'GAA region (giving ASO 660-Mut). This mutation entirely  
20 ablated TLR8 potentiation in HEK-TLR8 and THP-1 cells, confirming the importance of 5'end uridine residues in the potentiation of TLR8 by 2'OMe ASOs (Figure 9).

To further these results, the inventors tested the effect of the same strategy on ASO2 LNA, which the inventors previously found did not potentiate TLR8 (Fig. 1 - Alharbi et al., 2020). In this sequence context, addition of the 5'end 2'OMe UC motif  
25 (resulting in LNA ASO2-Mut1), failed to promote TLR8 potentiation. Similarly, 5' and 3' extension of ASO2 with 5'CUU and 3'UUC 2'OMe motifs (giving LNA ASO2-Mut2), did not influence TLR8 potentiation. This indicates that the strategy of 5'end modification, while able to confer TLR8 potentiation of 2'OMe ASOs, is not compatible with gapmer LNA ASOs. The inventors attribute this to the LNA modification  
30 potentially altering processing of the 5'end by a nuclease (see below - section on LNA ASO potentiation of TLR8) (Figure 9).

**Table 4:** Various oligonucleotides used in the below examples (all in 5'-3').  
UPPERCASE alone for DNA, 'm' indicates 2'OMe base, 'i2MOEr' indicates 2'MOE  
base, '+' indicates LNA base, and \* denotes the phosphorothioate backbone.

| Name             | Sequence                                                        | SEQ ID NO. |
|------------------|-----------------------------------------------------------------|------------|
| ASO1-UC          | mU*mC*mA*mU*mG*mG*mC*C*T*T*T*C*C*G*T*G*C*<br>mC*mA*mA*mG*mG     | 274        |
| ASO2 LNA         | +C*+G*+G*C*C*T*C*G*G*A*A*G*C*+T*+C*+T                           | 275        |
| ASO2-LNA<br>Mut1 | mU*mC*+C*+G*+G*C*C*T*C*G*G*A*A*G*C*+T*+C*+<br>T                 | 276        |
| ASO2-LNA<br>Mut2 | mC*mU*mU*+C*+G*+G*C*C*T*C*G*G*A*A*G*C*+T*+<br>C*+T*mU*mU*mC     | 277        |
| ASO 660          | mC*mU*mU*mC*mG*T*G*G*G*G*T*C*C*T*T*mU*mU*<br>mC*mA*mC           | 278        |
| ASO 660-Mut      | mG*mA*mA*mC*mG*T*G*G*G*G*T*C*C*T*T*mU*mU*<br>mC*mA*mC           | 279        |
| C2Mut-1          | mG*mC*mG*mG*mU*A*T*C*C*A*T*G*T*C*C*mC*mA<br>*mG*mG*mC           | 280        |
| C2Mut1-PS        | G*C*G*G*T*A*T*C*C*A*T*G*T*C*C*C*A*G*G*C                         | 281        |
| C2Mut1-2OMe      | mG*mC*mG*mG*mU*mA*mU*mC*mC*mA*mU*mG*mU<br>*mC*mC*mC*mA*mG*mG*mC | 282        |

**EXAMPLE 9: TLR8 potentiation is not limited to R848-like molecules**

Previous reports have demonstrated that R848-dependent activation of TLR8 is reliant on its binding to a site where uridine is normally binding (Tanji et al., 2015) (referred to as site 1). On the other hand, degradation products of uridine-containing  
5 RNAs are binding to a second site of the TLR8 dimer (site 2), generally as short di-nucleotides (e.g. UG or UUG or CG) (Tanji et al., 2015).

Uridine residues in the short RNAs binding to site 2 are not essential for TLR8 activation by uridine/R848 binding to site 1 as sensing of TLR8 by PS-ssRNA41 (lacking uridine) along with the TLR13 ligand Sa19 (with a single uridine residue) was  
10 potentiated with uridine (Shibata et al., 2016). Interestingly, PS-polyA, polyC or polyG failed to potentiate TLR8 sensing of uridine – aligning with the structural data and rather suggesting binding to selective RNA motifs (Shibata et al., 2016).

Speculating that the present 2'OMe ASOs (or their degradation products) potentiated R848 sensing by binding to site 2 of TLR8, the inventors next tested whether  
15 2'OMe ASOs could potentiate TLR8 sensing of free uridine. In agreement with this, the inventors observed a sequence-specific TLR8 potentiation of uridine sensing with select 2'OMe (ASO 660, 852dT) and dT20, in HEK-TLR8 (with NF-κB-luciferase) and THP-1 cells (with IP-10) (Figure 10). However, the 5'end mutant of ASO 660 failed to potentiate TLR8 sensing - mirroring the effects seen with R848, and confirming the  
20 sequence-dependent effect of ASOs.

The inventors have previously shown that ASO potentiation of R848 sensing resulted in increased IRF activation (presumably IRF5 through TASL recruitment). In agreement with this, ASO-potentiation of uridine sensing by TLR8 resulted in RANTES-luciferase induction (in a sequence-specific manner - compare ASO 660 and ASO 660-  
25 Mut) (Figure 10).

Collectively these results confirm that TLR8 potentiation by 2'OMe ASOs is not limited to synthetic imidazoquinoline compounds and is also visible with natural uridine (which binds to site 1 of TLR8).

Importantly, since the effect of ASO 660 on R848 and uridine potentiation is  
30 entirely ablated by the substitution of its 5' CUU motif with a GAA motif in ASO 660 Mut, the present results suggest that the short di-nucleotide required to potentiate TLR8 needs to originate from the 5'end of this ASO (with a similar finding for ASO1 and ASO1-UC). The importance of the CUU seen for potentiation in ASO 660 does not appear to be consistent with a role for RNase T2, which preferentially cleaved GU or AU  
35 motifs in ssRNAs (on both PS and phosphodiester backbone) (Greulich et al., 2019). Critically, the CUU motif in ASO 660 is in a 5' mCmUmU/mCmG context (all 2'OMe).

LNA ASO2 Mut2 also contains a 5' mCmUmU/+C+G context (where + is an LNA base). Since this does not result in TLR8 potentiation, the inventors speculate that the endonuclease necessary to release the CUU fragment is not effective in the context of an LNA base – noting that if the cleavage was operating at the 5' mCmU/mU position, there would not be a difference of TLR8 potentiation between the LNA ASO2 Mut2 and ASO 660, which both have this sequence.

#### **EXAMPLE 10: TLR8 potentiation by LNA and 2MOE gapmer ASOs**

The inventors previously demonstrated that gapmer ASO2 ASOs with LNA or 2'MOE modifications did not significantly potentiate TLR8 sensing of R848 (Fig. 1 - Alharbi et al., 2020). Based on the concept that these oligos are processed into fragments binding to site 2 of TLR8 to enhance uridine and R848 activity, these findings suggested that LNA and 2'MOE modifications hampered processing in the context of ASO2 (also noting that LNA ASO2 was only 16 nt and lacked 2 nt at 5' and 3' end).

To gain a broader insight into the effect of LNA and 2'MOE ASOs on TLR8 sensing, the inventors screened a panel of 91 LNA ASOs, and 76 2'MOE ASOs at 100 and 500 nM, in HEK-TLR8 cells treated with R848 (Figure 11, Table 5 and Table 6). For LNA ASOs, the inventors found that TLR8 potentiation was limited, with only 27% (25/91) of the ASOs leading to >2 fold increased NF-κB luciferase at 500 nM, with no ASO potentiating over 2 fold at 100 nM. This is in stark contrast with 2'OMe ASOs, for which >50% of the molecules potentiated TLR8 sensing by >2 fold at 100 nM (Fig. 2 - Alharbi et al., 2020).

For 2'MOE ASOs, the inventors found that TLR8 potentiation was greater than with LNA, but lesser than 2'OMe, with 50% (38/76) of the ASOs leading to >2 fold increased NF-κB luciferase at 500 nM, and with 34% (26/76) ASOs potentiating over 2 fold at 100 nM (Figure 11).

The inventors next sought to validate these results in repeat experiments in HEK TLR8 cells, using a few molecules from the screens. For LNA ASOs, the inventors confirmed that A7 and H11 significantly potentiated TLR8 sensing of R848 (Figure 12). However, when testing the same sequences in THP-1 cells, there was very limited potentiation compared to dT20 (~10 fold less) and A7 was the only sequence consistently increasing IP-10 production (H11 failed to do so in these cells) (Figure 12). While these results are consistent with a limited capacity of LNA ASOs to potentiate TLR8 sensing, they also indicate the possibility that the degradation pattern of the LNA ASOs could be cell-dependent (i.e. the endonuclease degrading ASO H11 in HEK cells may be absent in THP-1 cells).

The inventors also validated the results from the 2'MOE screen in a preliminary experiment in HEK-TLR8 cells (Figure 13). While this experiment only led to a modest TLR8 activation by R848 (which did not optimally activate the cells here), it validated the top potentiating ASOs (G9, D2, D7, B5, A9). Importantly, the inventors noted in the  
5 screen data that sequences with single nucleotide increments from the HPRT family exhibited different effects on TLR8. This was validated with this preliminary experiment, showing that ASO 663 but not 664/665/666 ASOs could potentiate TLR8 sensing. Although warranting further confirmation, this is of particular interest because the same ASO series made with 2'OMe chemistry showed a similar trend, with 2'OMe HPRT 663  
10 being the strongest TLR8 potentiator (663>664>665) (Fig. 5 - Alharbi et al., 2020).

Since the 2'OMe and 2'MOE gapmer ASOs the inventors used have the same length/sequence and only differ by the nature of the bases used in the 5' and 3' 5 nt regions, these results suggest that the pattern of endonuclease cleavage is conserved (sequence targeting by the nuclease is not prevented), although probably less efficient  
15 for 2'MOE ASOs compared to 2'OMe ASOs.

**Table 5 – TLR7 and TLR8 modulation by LNA ASOs**

| Well | SE  | Name                | Sequence                                            | TLR8<br>500nM | TLR8<br>100nM | TLR7<br>500nM | TLR7<br>100nM |
|------|-----|---------------------|-----------------------------------------------------|---------------|---------------|---------------|---------------|
| A1   | 283 | CTNN2B-311 LNA KL   | +C*+G*+C*+T*+T*+T*+C*+T*+G*+T*+C*+T*+G*+G*<br>+T    | 2.89068751    | 0.919880555   | 0.14185991    | 0.39318604    |
| A10  | 284 | HPRT LNA-665 V1     | +A*+C*+A*+C*+T*+T*+C*+G*+T*+G*+G*+G*+T*+C*<br>+C    | 1.028907862   | 0.421771896   | 0.08615711    | 0.54796744    |
| A11  | 285 | HPRT LNA-333 V1     | +T*+G*+A*+T*+G*+G*+C*+C*+T*+C*+C*+A*+T*+C*<br>+T    | 0.262861964   | 0.373839035   | 0.15275009    | 0.65650092    |
| A12  | 286 | HPRT LNA-330 V1     | +A*+T*+G*+G*+C*+C*+T*+C*+C*+C*+A*+T*+C*+C*<br>+C    | 1.355861146   | 0.368856109   | 0.11716763    | 0.55043319    |
| A2   | 287 | CTNN2B-1575 LNA V1  | +C*+C*+A*+C*+T*+T*+G*+G*+C*+A*+G*+A*+C*+C*+A<br>*+T | 1.212622978   | 0.683935484   | 0.46952034    | 0.76506387    |
| A3   | 288 | EGFR-2790 LNA KL    | +G*+T*+G*+A*+T*+C*+T*+T*+G*+A*+C*+A*+T*+G*+C*<br>+T | 1.806415165   | 0.583370618   | 0.06022367    | 0.43756054    |
| A4   | 289 | EGFR-1225 LNA V1    | +A*+G*+G*+C*+C*+C*+T*+T*+C*+G*+C*+A*+C*+T*+T*<br>+C | 0.967418317   | 0.508563128   | 0.16370864    | 0.73745623    |
| A5   | 290 | CAMSAP1-1635 LNA KL | +A*+C*+C*+A*+C*+G*+T*+C*+T*+G*+C*+T*+C*+T*+A*<br>+A | 4.259168003   | 0.933488362   | 0.53224953    | 1.07818714    |
| A6   | 291 | CAMSAP1-4915 LNA V1 | +G*+C*+A*+C*+A*+C*+T*+T*+C*+G*+T*+A*+C*+C*+C*<br>+A | 1.091460322   | 0.318981952   | 0.22110651    | 0.65677278    |

|     |     |                     |                                                    |             |             |            |            |
|-----|-----|---------------------|----------------------------------------------------|-------------|-------------|------------|------------|
| A7  | 292 | MB21D1-689 LNA KL   | +A*+A*+C*+C*+C*+C*+T*+T*+T*+C*+A*+C*+C*+A*+T*+C    | 8.653942007 | 1.871330912 | 0.42631118 | 0.77147456 |
| A8  | 293 | MB21D1-525 LNA V1   | +G*+A*+G*+G*+T*+C*+T*+T*+G*+G*+C*+T*+T*+C*+G*+T    | 0.641705492 | 0.573508016 | 0.06662907 | 0.79308061 |
| A9  | 294 | HPRT LNA-388 KL     | +C*+C*+A*+G*+C*+A*+G*+G*+T*+C*+A*+G*+C*+A*+A*+A    | 1.499436456 | 0.411543502 | 0.94211591 | 1.10083724 |
| B1  | 295 | CTNNB2-916 LNA KL   | +G*+A*+A*+A*+G*+G*+T*+T*+A*+T*+G*+C*+A*+A*+A*+G*+G | 4.109748834 | 1.156212285 | 0.10098192 | 0.16623017 |
| B10 | 296 | HPRT LNA-333 V1     | +G*+T*+G*+A*+T*+G*+G*+C*+C*+T*+C*+C*+A*+A*+T*+C    | 1.111733994 | 0.516764822 | 0.13894737 | 0.66514369 |
| B11 | 297 | HPRT LNA-335 V1     | +A*+T*+G*+T*+G*+A*+T*+G*+G*+C*+C*+T*+C*+C*+C*+C*+A | 1.839717337 | 0.637113907 | 0.12778074 | 0.49883046 |
| B12 | 298 | HPRT LNA-660 V1     | +T*+C*+G*+T*+G*+G*+G*+G*+T*+C*+C*+T*+T*+T*+T*+T*+C | 2.965725774 | 0.778445594 | 0.31788966 | 0.54005514 |
| B2  | 299 | CTNNB2-808 LNA V1   | +T*+G*+T*+C*+T*+G*+G*+A*+A*+G*+C*+T*+T*+C*+C*+C*+C | 0.871122112 | 0.600080893 | 0.27787327 | 0.49453053 |
| B3  | 300 | EGFR-3652 LNA KL    | +A*+G*+T*+G*+T*+G*+A*+G*+A*+T*+A*+C*+T*+T*+C*+G    | 0.599083955 | 0.427018507 | 0.11339072 | 0.55864079 |
| B4  | 301 | EGFR-4015 LNA V1    | +G*+T*+C*+C*+T*+T*+G*+C*+A*+C*+G*+T*+G*+G*+G*+C*+C | 0.935849795 | 0.570390577 | 0.25053206 | 0.47146409 |
| B5  | 302 | CAMSAP1-1739 LNA KL | +T*+A*+C*+T*+T*+C*+C*+G*+A*+G*+G*+T*+G*+T*+G*+T*+C | 1.585109868 | 0.417240354 | 0.4512019  | 0.73846497 |
| B6  | 303 | CAMSAP1-5594 LNA V1 | +G*+G*+C*+T*+C*+G*+G*+C*+T*+T*+G*+C*+C*+T*+A*+C    | 0.706148377 | 0.373742513 | 0.18619377 | 0.56938527 |

|     |     |                     |                                                       |             |             |            |            |
|-----|-----|---------------------|-------------------------------------------------------|-------------|-------------|------------|------------|
| B7  | 304 | MB21D1-719 LNA KL   | +G*+C*+A*C*+T*+C*A*G*+T*+C*+T*+G*+A*+G*+C             | 0.864230705 | 0.495364271 | 0.16817036 | 0.71281604 |
| B8  | 305 | MB21D1-523 LNA V1   | +G*+G*+T*+C*+T*+G*+G*+C*+T*+C*+G*+T*+G*+G*+G          | 1.385506297 | 0.616352872 | 0.04178645 | 0.48137976 |
| B9  | 306 | HPRT LNA-475 KL     | +G*+G*+T*+C*+A*+T*+A*+C*+A*+A*+T*+A*+G*+C*+T          | 1.380898459 | 0.49999171  | 0.04874837 | 0.57983939 |
| C1  | 307 | CTNN2B-1294 LNA KL  | +G*+A*+T*+A*+G*+C*+A*+C*+C*+T*+T*+C*+A*+G*+C*+A       | 2.862783595 | 0.820558594 | 0.04949276 | 0.43379199 |
| C10 | 308 | HPRT LNA-667 V1     | +C*+A*+A*+C*+A*+C*+T*+T*+C*+G*+T*+G*+G*+G*+G*+T       | 0.689536106 | 0.590755294 | 0.47631673 | 0.60256341 |
| C11 | 309 | HPRT LNA-334 V1     | +T*+G*+T*+G*+A*+T*+G*+G*+C*+C*+T*+C*+C*+C*+A*+A*+T    | 1.595846668 | 0.581865191 | 0.09010669 | 0.31544459 |
| C12 | 310 | HPRT LNA-669 V1     | +T*+C*+C*+A*+A*+C*+A*+C*+T*+T*+C*+G*+T*+G*+G*+G*+G    | 0.490756266 | 0.412801982 | 0.24944949 | 0.3182419  |
| C2  | 311 | CTNN2B-2545 LNA V1  | +C*+C*+A*+T*+C*+C*+A*+T*+G*+A*+G*+G*+T*+C*+C*+C*+T    | 1.168812131 | 0.674842397 | 0.64432531 | 0.99240168 |
| C3  | 312 | EGFR-3908 LNA KL    | +G*+T*+A*+T*+C*+G*+A*+A*+A*+G*+A*+G*+T*+C*+T*+C*+T    | 1.781012065 | 0.636606671 | 0.11343983 | 0.56243018 |
| C4  | 313 | EGFR-2874 LNA V1    | +G*+C*+C*+A*+T*+C*+C*+A*+C*+C*+T*+T*+G*+A*+T*+A*+A*+G | 0.681322005 | 0.440369427 | 0.12292291 | 0.67146076 |
| C5  | 314 | CAMSAP1-4295 LNA KL | +G*+G*+T*+T*+A*+C*+G*+G*+C*+T*+C*+A*+G*+T*+A*+A*+T    | 1.84782642  | 0.804379463 | 0.14008917 | 0.43785909 |
| C6  | 315 | CAMSAP1-4916 LNA V1 | +T*+G*+C*+A*+C*+A*+C*+T*+T*+C*+G*+T*+A*+C*+C*+C*+C    | 0.369671442 | 0.411275899 | 0.29939644 | 0.80645801 |

|     |     |                     |                                                             |             |             |            |            |
|-----|-----|---------------------|-------------------------------------------------------------|-------------|-------------|------------|------------|
| C7  | 316 | MB21D1-1181 LNA KL  | +T*+C*+G*+T*+A*G*+T*+G*+C*+T*+T*+G*+C*+T*+C*+C*+T*+<br>+A   | 1.582702591 | 0.556643844 | 0.19842456 | 0.38026667 |
| C8  | 317 | MB21D1-522 LNA V1   | +G*+T*+C*+T*+G*+G*+C*+T*+T*+C*+G*+T*+G*+G*+<br>+A           | 2.137051018 | 0.677910736 | 0.05637718 | 0.65162069 |
| C9  | 318 | HPRT LNA-489 KL     | +G*+T*+C*+C*+C*+C*+T*+G*+T*+T*+G*+A*+C*+T*+G*+<br>+G        | 0.303537628 | 0.3352112   | 0.12271748 | 0.55685117 |
| D1  | 319 | CTNN2B-2341 LNA KL  | +C*+C*+A*+A*+G*+A*+T*+C*+A*+G*+C*+A*+G*+T*+C*+<br>*+T       | 2.91994853  | 1.014820046 | 0.69656948 | 0.8818727  |
| D10 | 320 | HPRT LNA-329 V1     | +T*+G*+G*+C*+C*+T*+C*+C*+C*+A*+T*+C*+T*+C*+C*+<br>+T        | 1.127427243 | 0.495491714 | 0.21708902 | 0.87696501 |
| D11 | 321 | HPRT LNA-661 V1     | +T*+T*+C*+G*+T*+G*+G*+G*+T*+C*+G*+T*+C*+T*+T*+T*+<br>+T     | 3.144310898 | 0.620585256 | 0.17756599 | 0.48728787 |
| D12 | 322 | HPRT LNA-292 V1     | +G*+T*+T*+C*+A*+G*+T*+C*+C*+T*+G*+T*+C*+C*+C*+A*+<br>+T     | 4.128554096 | 1.068508653 | 0.11426897 | 0.45597999 |
| D2  | 323 | CTNN2B-1642 LNA V1  | +G*+C*+A*+G*+G*+C*+T*+C*+A*+G*+T*+G*+A*+T*+G*+<br>*+T       | 1.439533955 | 0.638180016 | 0.18884129 | 0.67647223 |
| D3  | 324 | EGFR-4705 LNA KL    | +G*+C*+C*+T*+G*+G*+A*+A*+T*+C*+C*+G*+A*+G*+T*+<br>*+T       | 0.577088592 | 0.561637084 | 0.04131333 | 0.41656083 |
| D4  | 325 | EGFR-3267 LNA V1    | +G*+C*+A*+T*+C*+A*+C*+C*+A*+C*+C*+A*+C*+G*+T*+C*+G*+<br>*+T | 0.738233723 | 0.607383649 | 0.08520819 | 0.55525442 |
| D5  | 326 | CAMSAP1-4697 LNA KL | +T*+T*+T*+T*+G*+G*+C*+T*+G*+G*+G*+A*+T*+C*+A*+<br>+A        | 1.411677311 | 0.542417088 | 0.30542713 | 0.56354698 |
| D6  | 327 | CAMSAP1-2584 LNA V1 | +G*+C*+C*+A*+T*+G*+C*+T*+G*+G*+C*+T*+C*+G*+G*+<br>*+A       | 0.363474708 | 0.446408939 | 0.17542771 | 0.6380256  |

|     |     |                     |                                               |             |             |            |            |
|-----|-----|---------------------|-----------------------------------------------|-------------|-------------|------------|------------|
| D7  | 328 | MB21D1-1248 LNA KL  | +G*+C*+C*A*+T*G*+T*+T*+C*+T*+T*+C*+T*+T*+T*+G | 0.431182656 | 0.430459006 | 0.02075046 | 0.81600703 |
| D8  | 329 | MB21D1-188 LNA V1   | +C*+G*+G*+C*+C*+T*+C*+G*+A*A*+G*+C*+T*+C      | 0.21857614  | 0.401507928 | 0.1389126  | 0.59401711 |
| D9  | 330 | HPRT LNA-551 KL     | +T*+C*+C*A*+C*A*+T*+C*A*+A*+G*A*+C*+A         | 0.89344393  | 0.474275592 | 0.53522126 | 0.86313802 |
| E1  | 331 | CTNNB2B-3648 LNA KL | +C*+T*+T*+G*A*A*+G*+C*A*+T*+C*+G*+T*+A*+T*    | 3.888648608 | 0.959918056 | 0.0438927  | 0.36286553 |
| E10 | 332 | HPRT LNA-666 V1     | +A*+A*+C*A*+C*+T*+C*+G*+T*+G*+G*+G*+T         | 0.882130479 | 0.496305985 | 0.44467195 | 0.89893562 |
| E11 | 333 | HPRT LNA-662 V1     | +C*+T*+T*+C*+G*+T*+G*+G*+G*+T*+C*+C*+T*+T*    | 4.366497579 | 1.097412417 | 0.23301734 | 0.68538279 |
| E12 | 334 | HPRT LNA 898 999    | +A*+G*+G*+A*+C*+T*+C*+A*+G*A*+T*+G*+T*+T      | 1.034353241 | 0.278472837 | 0.29310899 | 0.43663496 |
| E2  | 335 | CTNNB2B-2516 LNA V1 | +A*+T*+C*+T*+G*+G*+C*A*+G*+C*+C*+A*+T*+C*     | 2.628295796 | 0.807587707 | 0.27890078 | 0.66285286 |
| E3  | 336 | EGFR-488 LNA KL     | +A*+G*+A*+T*+T*+C*+A*+G*+A*+G*+C*+A*+G*+C     | 0.77018825  | 0.401114395 | 0.0206294  | 0.43772578 |
| E4  | 337 | EGFR-1013 LNA V1    | +C*+C*+T*+T*+G*+C*A*+C*+G*+T*+G*+G*+T*+T*     | 2.414242522 | 1.314579837 | 0.28128379 | 0.6172155  |
| E5  | 338 | CAMSAP1-6273 LNA KL | +A*+A*+A*+G*+G*+A*+C*+T*+G*A*+G*+G*A*+A*+A    | 2.662700169 | 0.881982649 | 0.26740807 | 0.51270535 |
| E6  | 339 | CAMSAP1-2709 LNA V1 | +T*+T*+C*A*+G*+G*+C*+G*+C*+T*+G*+C*+T*+T*     | 0.764329236 | 0.478887514 | 0.18055434 | 0.6416398  |

|     |     |                        |                                                      |             |              |            |            |
|-----|-----|------------------------|------------------------------------------------------|-------------|--------------|------------|------------|
| E7  | 340 | MB21DI-1483 LNA KL     | +T*+G*+A*+C*+T*G*+T*G*+T*G*+A*G*+G*+G*+<br>+T        | 3.209604677 | 0.9725735    | 0.14999726 | 0.80240327 |
| E8  | 341 | MB21DI-520 LNA V1      | +C*+T*+T*G*G*+C*+T*G*+T*G*+A*+G*+<br>+C              | 0.594607406 | 0.458104857  | 0.09794609 | 0.80632843 |
| E9  | 342 | HPRT LNA-692 KL        | +A*+A*+T*+C*+A*+A*+C*+A*+A*+A*+G*+T*+C*+T*<br>*+G    | 1.899545122 | 0.390751475  | 0.4326279  | 0.72655691 |
| F1  | 343 | CTNNB-809 LNA V1       | +G*+T*+G*+T*+C*+T*G*+G*+A*+A*+G*+C*+T*+T*+C*<br>+C   | 2.057291617 | 0.922865713  | 0.0668831  | 0.3554078  |
| F10 | 344 | HPRT LNA-664 V1        | +C*+A*+C*+T*+C*+T*G*+T*G*+G*+G*+T*+C*+C*+<br>+T      | 1.446145761 | 0.424556089  | 0.24003633 | 0.86814066 |
| F11 | 345 | HPRT LNA-331 V1        | +G*+A*+T*+G*+C*+C*+T*+C*+T*+C*+A*+T*+C*+T*<br>+C     | 0.185913879 | 0.278338605  | 0.11903826 | 0.55812929 |
| F12 | 346 | NC1 LNA PS 3-10-3      | +C*+G*+T*+A*+T*+T*+A*+T*+A*+G*+C*+G*+A*+T*+<br>+T    | 0.716037601 | 0.396086198  | 0.22566078 | 0.75412488 |
| F2  | 347 | CTNNB-2136 LNA V1      | +C*+T*+G*+C*+A*+G*+C*+T*+T*+C*+C*+T*+T*+G*+T*+<br>+C | 0.935994958 | 0.477630621  | 0.05267412 | 0.4668655  |
| F3  | 348 | EGFR-1014 LNA V1       | +T*+C*+C*+T*+G*+C*+A*+C*+G*+T*+G*+G*+C*+T*+<br>+T    | 0.601229377 | 0.491207464  | 0.48221215 | 0.81167637 |
| F4  | 349 | EGFR-1016 LNA V1       | +T*+G*+T*+C*+C*+T*+T*+G*+C*+A*+C*+G*+T*+G*+G*+<br>+C | 1.157848602 | 0.6355917805 | 0.19903806 | 0.50221199 |
| F5  | 350 | CAMSAP1-2112 LNA<br>V1 | +T*+C*+T*+T*+C*+A*+T*+C*+G*+G*+C*+C*+T*+G*+<br>+C    | 1.393172852 | 0.595094771  | 0.51085066 | 0.81989598 |
| F6  | 351 | CAMSAP1-2456 LNA<br>V1 | +G*+T*+C*+T*+C*+T*+G*+G*+A*+G*+C*+T*+T*+C*+C*+<br>+T | 2.401950564 | 0.764434294  | 0.08440541 | 0.47710507 |

|     |     |                     |                                                                                                                                                                                                                          |             |             |            |            |
|-----|-----|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|-------------|------------|------------|
| F7  | 352 | MB21D1-521 LNA V1   | +T*+C*+T* <sup>+</sup> T*G*G* <sup>+</sup> C* <sup>+</sup> T* <sup>+</sup> T* <sup>+</sup> C* <sup>+</sup> G* <sup>+</sup> T* <sup>+</sup> G* <sup>+</sup> +A* <sup>+</sup>                                              | 0.196973894 | 0.5405367   | 0.28768075 | 1.0325815  |
| F8  | 353 | MB21D1-616 LNA V1   | +A*+G*+C* <sup>+</sup> T* <sup>+</sup> T* <sup>+</sup> C* <sup>+</sup> G* <sup>+</sup> A* <sup>+</sup> G* <sup>+</sup> C* <sup>+</sup> C* <sup>+</sup> +C* <sup>+</sup> +A* <sup>+</sup>                                 | 0.864508317 | 0.331485279 | 0.11099721 | 0.55425805 |
| F9  | 354 | HPRT LNA-732 KL     | +G*+T*+C* <sup>+</sup> A* <sup>+</sup> A* <sup>+</sup> G* <sup>+</sup> G* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> T* <sup>+</sup> A* <sup>+</sup> T* <sup>+</sup> +C* <sup>+</sup> +C* <sup>+</sup>                 | 2.301162835 | 0.543243053 | 0.14162734 | 0.65040068 |
| G1  | 355 | CTNNB-2446 LNA V1   | +T*+C*+C* <sup>+</sup> A* <sup>+</sup> T* <sup>+</sup> A* <sup>+</sup> C* <sup>+</sup> C* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> A* <sup>+</sup> G* <sup>+</sup> +C* <sup>+</sup> +A* <sup>+</sup>                 | 1.334008584 | 0.595203037 | 0.49364331 | 1.05617457 |
| G10 | 356 | HPRT LNA-668 V1     | +C*+C*+A* <sup>+</sup> A* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> C* <sup>+</sup> T* <sup>+</sup> T* <sup>+</sup> C* <sup>+</sup> G* <sup>+</sup> T* <sup>+</sup> G* <sup>+</sup> +G* <sup>+</sup> +G* <sup>+</sup> | 0.835058763 | 0.534877754 | 0.4460221  | 0.80831707 |
| G11 | 357 | HPRT LNA-294 V1     | +A*+C*+G* <sup>+</sup> T* <sup>+</sup> T* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> G* <sup>+</sup> T* <sup>+</sup> C* <sup>+</sup> T* <sup>+</sup> G* <sup>+</sup> +T* <sup>+</sup> +C* <sup>+</sup>                 | 0.49347248  | 0.356093832 | 0.13623624 | 0.60093638 |
| G12 | 358 | NC5 LNA PS 3-10-3   | +G*+A*+C* <sup>+</sup> T* <sup>+</sup> A* <sup>+</sup> T* <sup>+</sup> A* <sup>+</sup> C* <sup>+</sup> G* <sup>+</sup> C* <sup>+</sup> G* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> +A* <sup>+</sup> +T* <sup>+</sup> | 3.375696066 | 0.777122568 | 0.09759621 | 0.2817351  |
| G2  | 359 | CTNNB-2479 LNA V1   | +T*+G*+G* <sup>+</sup> T* <sup>+</sup> G* <sup>+</sup> C* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> C* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> +T* <sup>+</sup> +C* <sup>+</sup>                                 | 0.604463097 | 0.463590487 | 0.39295731 | 0.9568601  |
| G3  | 360 | EGFR-3566 LNA V1    | +C*+A* <sup>+</sup> T* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> C* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> C* <sup>+</sup> G* <sup>+</sup> T* <sup>+</sup> C* <sup>+</sup> +G* <sup>+</sup> +T* <sup>+</sup>    | 2.417304533 | 0.77840921  | 0.35533763 | 0.74095298 |
| G4  | 361 | EGFR-727 LNA V1     | +G*+T*+C* <sup>+</sup> C* <sup>+</sup> G* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> C* <sup>+</sup> G* <sup>+</sup> T* <sup>+</sup> G* <sup>+</sup> +A* <sup>+</sup> +T* <sup>+</sup>                                 | 0.744775878 | 0.585159169 | 0.31080227 | 0.84335141 |
| G5  | 362 | CAMSAP1-2113 LNA V1 | +G*+T*+C* <sup>+</sup> T* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> T* <sup>+</sup> C* <sup>+</sup> G* <sup>+</sup> C* <sup>+</sup> C* <sup>+</sup> +C* <sup>+</sup> +T* <sup>+</sup>                                 | 2.086214626 | 0.663716035 | 0.11859632 | 0.64096423 |
| G6  | 363 | CAMSAP1-2906 LNA V1 | +C*+C*+A* <sup>+</sup> C* <sup>+</sup> A* <sup>+</sup> T* <sup>+</sup> C* <sup>+</sup> T* <sup>+</sup> G* <sup>+</sup> T* <sup>+</sup> G* <sup>+</sup> +C* <sup>+</sup> +T* <sup>+</sup>                                 | 0.232442282 | 0.291365866 | 0.36345034 | 0.78888908 |

|     |     |                        |                                                      |             |             |            |            |
|-----|-----|------------------------|------------------------------------------------------|-------------|-------------|------------|------------|
| G7  | 364 | MB21D1-524 LNA V1      | +A*+G*+G*+T*+C*+T*+G*+G*+C*+T*+C*+G*+T*+<br>+G       | 1.933232965 | 0.596247374 | 0.05003212 | 0.45714258 |
| G8  | 365 | MB21D1-697 LNA V1      | +T*+G*+G*+T*+C*+C*+A*+C*+A*+C*+C*+C*+T*+<br>+T       | 1.433569312 | 0.558507437 | 0.54529597 | 1.00843749 |
| G9  | 366 | HPRT LNA-1027 KL       | +T*+C*+C*+G*+C*+C*+A*+A*+G*+G*+A*+A*+<br>*+C         | 0.724923765 | 0.540879865 | 0.54271738 | 0.86013861 |
| H1  | 367 | CTNNB2-1576 LNA V1     | +C*+C*+C*+A*+C*+T*+G*+G*+C*+A*+G*+A*+C*+C*+<br>*+A   | 1.865354601 | 0.898697761 | 0.63913972 | 1.02415107 |
| H10 | 368 | HPRT LNA-663 V1        | +A*+C*+T*+T*+C*+G*+T*+G*+G*+G*+T*+C*+C*+T*+<br>+T    | 3.672132066 | 0.819821852 | 0.73554308 | 0.80396669 |
| H11 | 369 | HPRT LNA-326 V1        | +C*+C*+T*+C*+C*+A*+T*+C*+T*+C*+C*+T*+T*+C*+<br>+A    | 6.75898779  | 1.46930113  | 0.84318021 | 0.75846825 |
| H3  | 370 | EGFR-2859 LNA V1       | +G*+G*+C*+A*+C*+T*+T*+G*+C*+C*+T*+C*+C*+C*+T*+<br>+T | 0.862488571 | 0.651786464 | 0.66841118 | 0.77430522 |
| H5  | 371 | CAMSAP1-1850 LNA<br>V1 | +T*+G*+C*+T*+C*+C*+T*+C*+G*+T*+C*+T*+C*+C*+<br>+C    | 4.77079461  | 1.097052718 | 0.59461451 | 0.75110463 |
| H7  | 372 | MB21D1-526 LNA V1      | +G*+G*+A*+G*+G*+T*+C*+T*+G*+G*+C*+T*+T*+C*+<br>+G    | 0.882197694 | 0.574984194 | 0.11943028 | 0.5030592  |
| H9  | 373 | HPRT LNA-1353 KL       | +T*+G*+A*+C*+A*+A*+G*+A*+T*+T*+C*+A*+C*+T*+<br>*+G   | 1.756020752 | 0.633203821 | 0.42963559 | 1.04030631 |
|     |     |                        | NT                                                   | 0.022710362 | 0           | 0          | 0          |
|     |     |                        | NT                                                   | 0           | 0.004817336 | 0.0112819  | 0.03133982 |

**Table 6 – TLR7 and TLR8 modulation by 2'MOE ASOs**

[illegible]

[illegible]

[illegible]



[illegible]

[illegible]

|      |    |                      |                   |                                                                                                                                             |                 |                 |                |                |
|------|----|----------------------|-------------------|---------------------------------------------------------------------------------------------------------------------------------------------|-----------------|-----------------|----------------|----------------|
| G 5  | 43 | EGFR-3266<br>MOE     | 1648<br>6364<br>9 | /52MOE/G*/12MOE/G*/12MOE/A*/12MOE/H*/12MOE/T*/C*/A*/C*/C*/A*/<br>C*/G*/T*/C*/12MOE/G*/12MOE/T*/12MOE/C*/12MOE/C*/12MOE/A/                   | 1.5658<br>7114  | 1.6185<br>06125 | 0.0971<br>6989 | 0.2157<br>6031 |
| G 6  | 43 | EGFR-727<br>MOE      | 1648<br>6365<br>7 | /52MOE/A*/12MOE/T*/12MOE/G*/12MOE/H*/12MOE/C*/C*/G*/C*/A*/<br>C*/T*/G*/12MOE/A*/12MOE/T*/12MOE/G*/12MOE/H*/12MOE/C*/12MOE/T/                | 1.7079<br>82031 | 2.0287<br>68596 | 0.1708<br>5396 | 0.3971<br>7936 |
| G 7  | 44 | CAMSAP1<br>-2113 MOE | 1648<br>6366<br>4 | /52MOE/G*/12MOE/T*/12MOE/G*/12MOE/H*/12MOE/C*/T*/C*/A*/T*/C*/<br>G*/G*/C*/12MOE/C*/12MOE/T*/12MOE/G*/12MOE/H*/12MOE/C*/12MOE/H/             | 1.0933<br>44915 | 1.2471<br>02513 | 0.1319<br>9722 | 0.3712<br>7496 |
| G 8  | 44 | CAMSAP1<br>-2906 MOE | 1648<br>6367<br>2 | /52MOE/G*/12MOE/T*/12MOE/C*/12MOE/H*/12MOE/C*/C*/A*/T*/C*/T*/<br>G*/T*/G*/12MOE/C*/12MOE/T*/12MOE/H*/12MOE/C*/12MOE/G*/12MOE/H/             | 0.4928<br>97094 | 0.9187<br>50444 | 0.1189<br>2947 | 0.2989<br>7614 |
| G 9  | 44 | MB21D1-<br>524 MOE   | 1648<br>6367<br>9 | /52MOE/G*/12MOE/G*/12MOE/A*/12MOE/H*/12MOE/G*/12MOE/H*/T*/C*/T*/G*/G*/<br>C*/T*/C*/12MOE/G*/12MOE/T*/12MOE/H*/12MOE/G*/12MOE/H*/12MOE/A/    | 5.2877<br>57611 | 3.9753<br>20288 | 0.0912<br>4819 | 0.4337<br>1803 |
| H 1  | 44 | HPRT-329<br>MOE      | 1648<br>6362<br>1 | /52MOE/G*/12MOE/A*/12MOE/T*/12MOE/G*/12MOE/H*/C*/C*/T*/C*/C*/C*/<br>A*/T*/C*/12MOE/C*/12MOE/C*/12MOE/T*/12MOE/H*/12MOE/T*/12MOE/H/          | 1.6980<br>23797 | 0.9786<br>70686 | 0.3360<br>5342 | 0.6776<br>8814 |
| H 10 | 44 | NC5<br>2MOE          | 1576<br>3543<br>9 | /52MOE/G*/12MOE/C*/12MOE/G*/12MOE/A*/12MOE/H*/12MOE/C*/T*/A*/A*/C*/G*/<br>C*/G*/C*/A*/12MOE/A*/12MOE/T*/12MOE/H*/12MOE/A*/12MOE/H*/12MOE/H/ | 0.8830<br>39592 | 1.0671<br>59916 | 0.2942<br>2227 | 0.9481<br>7716 |
| H 3  | 44 | CTNNB2B-<br>1576 MOE | 1648<br>6363<br>5 | /52MOE/C*/12MOE/A*/12MOE/C*/12MOE/H*/12MOE/C*/A*/C*/T*/G*/G*/<br>C*/A*/G*/A*/12MOE/C*/12MOE/H*/12MOE/A*/12MOE/H*/12MOE/H/                   | 1.5030<br>65697 | 1.4404<br>08915 | 0.2270<br>2965 | 0.6004<br>9629 |
| H 5  | 44 | EGFR-2859<br>MOE     | 1648<br>6365<br>0 | /52MOE/T*/12MOE/A*/12MOE/G*/12MOE/H*/12MOE/C*/A*/C*/T*/T*/G*/<br>C*/C*/T*/C*/12MOE/C*/12MOE/T*/12MOE/H*/12MOE/C*/12MOE/H/                   | 0.8844<br>82754 | 0.7727<br>87531 | 0.2051<br>5584 | 0.4550<br>184  |
| H 7  | 44 | CAMSAP1<br>-1850 MOE | 1648<br>6366<br>5 | /52MOE/G*/12MOE/A*/12MOE/T*/12MOE/G*/12MOE/H*/12MOE/C*/T*/C*/T*/C*/G*/<br>G*/T*/C*/T*/12MOE/C*/12MOE/C*/12MOE/C*/12MOE/H*/12MOE/T*/12MOE/H/ | 1.9537<br>62836 | 1.3917<br>56007 | 0.1993<br>1744 | 0.4525<br>4896 |
| H 8  | 44 | NC1<br>2MOE          | 1576<br>3543<br>8 | /52MOE/G*/12MOE/C*/12MOE/G*/12MOE/H*/12MOE/A*/T*/T*/A*/T*/A*/G*/<br>C*/C*/G*/A*/12MOE/T*/12MOE/H*/12MOE/A*/12MOE/H*/12MOE/H/                | 2.7769<br>93481 | 1.8830<br>52499 | 0.2649<br>1771 | 0.5652<br>492  |

| H | 44<br>9 | NB2ID1-<br>526 MOE | 1648<br>6368<br>0 | $\frac{1}{2} \text{MOEG} * \frac{1}{2} \text{MOEC} * \frac{1}{2} \text{MOEG} * \frac{1}{2} \text{MOEA} * \text{G}^* \text{G}^* \text{T}^* \text{C}^* \text{T}^* \text{T}^*$<br>$\text{G}^* \text{G}^* \text{C}^* \text{T}^* \frac{1}{2} \text{MOET}^* \frac{1}{2} \text{MOEC}^* \frac{1}{2} \text{MOEG}^* \frac{1}{2} \text{MOET}^* \frac{1}{2} \text{MOEC}^*$ | 2,2764<br>36415 | 1,5981<br>5863  | 0,1535<br>6207 | 0.4525<br>2984 |
|---|---------|--------------------|-------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|-----------------|----------------|----------------|
| G | 11      | NT                 |                   |                                                                                                                                                                                                                                                                                                                                                                | 0,0598<br>47661 | 0,0249<br>62066 | 0,0144<br>466  | 0              |
| G | 12      | NT                 |                   |                                                                                                                                                                                                                                                                                                                                                                | 0,0368<br>60718 | 0,0134<br>97566 | 0,0169<br>8715 | 0,0028<br>5387 |
| H | 11      | NT                 |                   |                                                                                                                                                                                                                                                                                                                                                                | 0,1433<br>565   | 0,0887<br>29649 | 0,0084<br>9868 | 0,0488<br>9513 |
| H | 12      | NT                 |                   |                                                                                                                                                                                                                                                                                                                                                                |                 | 0,0182          | 0,0040         | 0,0040         |
|   |         |                    |                   |                                                                                                                                                                                                                                                                                                                                                                | 0               | 18026           | 0              | 5533           |

**EXAMPLE 11: TLR8 potentiation requires co-administration of site 1 and site 2 ligands**

In all the experiments to date, the inventors used a pre-treatment of the cells with their ASOs (~30 min in HEKs – overnight in THP-1 cells), prior to R848 stimulation.

- 5 Critically, the inventors always kept the ASOs in the supernatants during uridine or R848 stimulation. To better define whether *de novo* degradation of the oligonucleotides was required for the effect on R848, the inventors pre-incubated PS-dT20 with HEK-TLR8 cells, and then washed them off prior to R848 stimulation (Figure 14). Surprisingly, removal of dT20 from the supernatants at the time of R848 stimulation strongly  
10 decreased TLR8 potentiation by this oligonucleotide. This indicates that dT20 is rapidly degraded once in the endosome, and that its degradation products, presumably binding to site 2 of TLR8, are very short lived and essential to potentiate R848 activity.

It is also noteworthy that the inventors noticed short incubation of their 2'OMe ASOs in THP-1 (~2.5 h) strongly decreased their potentiating capacity compared to  
15 overnight incubation, but that this did not alter the effect of dT20 (Figure 14). Based on the observation that the effect of dT20 does not persist after wash-off in HEKs, the inventors speculate that degradation of their 2'OMe ASOs is probably slower than that of dT20 (thereby explaining why their effect is reduced with the 2.5h pre-incubation compared to dT20).

- 20 Collectively, these results suggest a negative correlation between ASO stability and TLR8 potentiation that requires further studies. Since 2'MOE potentiation of the HPRT 663-665 series appears to be similar to that of the 2'OMe, it indicates that 2'MOE ASOs are still processed in a similar fashion as 2'OMe (probably by the same nuclease), but probably less rapidly. This is consistent with the concept that 2'MOE ASOs are  
25 usually more potent at promoting mRNA down-regulation than 2'OMe ASOs – potentially partially relating to greater intracellular stability.

This could provide opportunities for the selection and design of ASOs potentiating TLR8 sensing of uridine or R848 for longer times – which would be of particular importance when the ASO and R848/uridine/site 1 agonist are not  
30 administered at the same time.

**EXAMPLE 12: TLR8 potentiation of co-cultured cells – and implications in cancer**

Next, the inventors were interested to test whether a cell transfected with one of their 2'OMe ASOs potentiating TLR8 (using 852dT as a model), when co-cultured with  
35 phagocytes, could potentiate TLR8 sensing in phagocytes. The inventors reasoned that unprocessed ASOs or their degradation products could favor R848 sensing of TLR8, on

the basis of a recent publication that showed that phagocytosis of apoptotic cells transfected with synthetic PS- modified DNA molecules resulted in phago-lysosomal delivery of the DNA in the phagocytes (Ahn et al., 2018).

Here the inventors transfected HEK cells with 2'OMe ASO3 (non TLR8  
5 potentiating) or 852dT (strongly potentiating TLR8), prior to UV treatment and co-culture with PMA-differentiated THP-1 overnight, before 24 h R848 stimulation (Figure 15). In this set up, R848 stimulation of co-cultures with ASO3 transfected HEK cells only marginally upregulated TNF $\alpha$  production (2.8 fold), while R848 stimulation of co-cultures with 852dT transfected HEK cells strongly up-regulated TNF $\alpha$  production (>13  
10 fold) (Figure 15).

These results suggest that the intracellular ASOs in HEKs (or their degradation products), found their way to the endosome of THP-1 cells to result in potentiation of TLR8 sensing.

This observation could have implications in the context of tumor targeting ASOs.  
15 As such, bi-functional ASOs with cancer cell killing activity and TLR8 potentiating activity, could also be taken up (or their degradation products) by closely surrounding phagocytes. When stimulated with uridine/R848 site 1 ligands, these tumor phagocytes could end up being strongly activated to promote recruitment of further immune cells, while also favoring MHC presentation of cancer cell peptides they would have engulfed.  
20 Critically, tumour phagocytes that are not directly engaged in phagocytosis of dying cancer cells (and do not have endosomal ASO degradation products and cancer epitopes to present) would not be strongly activated by R848.

#### **EXAMPLE 13: TLR8 potentiation with fully 2'OMe ASO**

25 The inventors also tested the capacity of fully 2'OMe-modified ASOs (with no central DNA « gap ») to potentiate TLR8. For this, the inventors compared the effect of ASO C2Mut1, and its variants either fully lacking 2'OMe (referred to as C2Mut1-PS), or fully 2'OMe modified (C2Mut1-2OMe). These experiments showed that even in the absence of a central DNA region, this ASO was still significantly potentiating TLR8  
30 (noting that this family of ASOs was not a strong potentiator compared to other sequences) (Figure 16). Critically these results suggest that fully 2'OMe oligonucleotides can be spontaneously taken up by cells to activate endosomal TLR8, without the need for transfection.

#### **35 EXAMPLE 14: TLR7 inhibition – modulation by 5'end motifs**

The inventors also tested the mutant sequences (ASO1-UC, LNA ASO2-mut1 and mut2, and ASO 660/ASO 660-Mut) on TLR7 sensing of R848. In accord with their previous finding that 5' terminal CUU motifs were important to limit TLR7 inhibition by 2'OMe ASOs, ASO 660-Mut (lacking its 5' end mCmUmU motif) was significantly more inhibitory than ASO-660 (Figure 17). Conversely, LNA ASO2 mut2 (also harboring a 5' mCmUmU motif) retained TLR7 inhibition. Addition of a 5'UC motif in ASO1-UC did not alter TLR7 inhibition (while it did strongly increase TLR8 potentiation).

10 **EXAMPLE 15: TLR7 inhibition – modulation by base modifications**

The inventors have previously observed that ASO2 LNA or 2'MOE variants were strongly inhibiting TLR7 sensing (Fig. 1, Alharbi et al., 2020). To define whether select motifs in LNA or 2'MOE ASOs would preclude TLR7 inhibition, the inventors tested their panels of 91 LNA ASOs, and 76 2'MOE ASOs at 100 and 500 nM, in HEK-TLR7 cells treated with R848 (Figure 18).

For LNA ASOs, the inventors found that TLR7 inhibition was predominant, with only 85% (78/91) of the ASOs leading to 50% decreased NF-κB luciferase at 500 nM, and 52% (48/91) of the ASOs leading to 80% decreased NF-κB luciferase at that dose. Such TLR7 inhibition was however a bit less frequent than what the inventors observed for 2'OMe, for which >78% of the molecules inhibited TLR7 sensing by 80% fold at 500 nM (Fig. 2 - Alharbi et al., 2020).

For 2'MOE, the inventors found 95% (72/76) of the ASOs leading to 50% decreased NF-κB luciferase at 500 nM, and 54% (41/76) of the ASOs leading to 80% decreased NF-κB luciferase at that dose (Figure 18). Critically, however, a similar to what the inventors saw for 2'OMe, a few select ASOs did not inhibit TLR7 (e.g. A9, H11, D1, H1 for LNA, and G1, C2, C1, A9, A2 for 2'MOE).

The inventors next sought to validate these results in repeat experiments, focusing on the ASOs that did not inhibit TLR7. Specifically, the inventors noted that the LNA ASOs A9, H11, D1, H1 all had a 5' +C+C motif (+ denotes LNA modification) – and that such motif was absent in all the strong TLR7 inhibitor (i.e. with > 75% inhibition at 500 nM). The inventors also included A11, B1 and C11 that inhibited TLR7. Repeat experiments confirmed the trend of the screen, validating that LNA D1 and H11 did not significantly decrease NF-κB luciferase activity (noting that A9 rather slightly increased NF-κB luciferase, and H1 only mildly reduced it) (Figure 19).

For 2'MOE ASOs, the inventors only ran a single preliminary experiment that confirmed the decreased TLR7 inhibition with G1, A2, C1 and A9 (compared to the other

ASO tested which, in this experiment, entirely ablated TLR7 signalling). Importantly, the inventors noted that sequences with single nucleotide increments E1 (665), A2 (666), and G1 (667) exhibited different effects on TLR7. As such, only A2 and G1 2'OMe ASOs had reduced TLR7 activity (suggesting the existence of a motif regulating TLR7 inhibition, similar to what the inventors found for 2'OMe ASOs). While warranting further confirmation, this is of particular interest because the same ASO series made with 2'OMe chemistry differently inhibited TLR7 – 666 and 667 2'OMe ASOs inhibited more TLR7 than 665 (Fig. 5 - Alharbi et al., 2020)(Figure 19).

Collectively these results confirm that some sequences have the capacity to be less immunosuppressive on TLR7 than others with all three gapmer ASO chemistries.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

All publications discussed and/or referenced herein are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

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