The present invention provides 4-nucleotide (4mer) RNA motifs that confer immunostimulatory activity, in particular, IFN-α-inducing activity to a RNA oligonucleotide. The present invention also provides RNA oligonucleotides, including siRNA, with high or low immunostimulatory activity. The present invention further provides the use of the RNA oligonucleotides of the invention for therapeutic purposes.
Field of the Invention

The present invention relates to the field of immunotherapy and drug discovery. The present invention provides a method for determining the immunostimulatory activity of a RNA oligonucleotide. The present invention also provides a method for predicting the immunostimulatory activity of a RNA oligonucleotide. The present invention further provides a method for preparing RNA oligonucleotides with high or low immunostimulatory activity. Moreover, the present invention provides RNA oligonucleotides with immunostimulatory activity and the therapeutic uses thereof. In addition, the present invention provides RNA oligonucleotides with gene silencing activity and with either high or low immunostimulatory activity, the methods of their preparation, and their therapeutic uses.

Background of the Invention

The vertebrate immune system established different ways to detect invading pathogens based on certain characteristics of their microbial nucleic acids. Detection of microbial nucleic acids alerts the immune system to mount the appropriate type of immune response that is required for the defense against the respective type of pathogen detected. Detection of viral nucleic acids leads to the production of type I interferon (IFN), the key cytokine for anti-viral defence. An understanding of how nucleic acids interact with the vertebrate immune system is important for developing different nucleic acid-based therapeutic strategies for the immunotherapy of diseases (Rothenfusser S et al. 2003, Curr Opin Mol Ther 5:98-106) and for developing gene-specific therapeutic agents (Tuschl T et al. 2002, Mol Inten/ 2: 158-167).


IFN-α was the first type of interferon to be identified and commercialized; it is widely used clinically in the treatment of a variety of tumors (e.g., hairy cell leukemia, cutaneous T cell leukemia, chronic myeloid leukemia, non-Hodgkin's lymphoma, AIDS-related Kaposi's sarcoma, malignant melanoma, multiple myeloma, renal cell carcinoma, bladder cell carcinoma, colon carcinoma, cervical dysplasia) and viral diseases (e.g., chronic hepatitis B, chronic hepatitis C). IFN-α products that are currently in clinical use include the recombinant protein and the highly purified natural protein, both of which have high production costs. Therefore, there is a need for more economical ways of providing IFN-α to patients in need. Furthermore, IFN-α is currently administrated systemically and causes a broad spectrum of side effects (e.g. fatigue, flu-like symptoms, diarrhea). Most alarmingly, IFN-α causes a decrease in bone marrow function which leads to increased susceptibility to life-threatening infections, anemia and bleeding problems. Therefore, there is a need for ways of providing IFN-α in a more localized (i.e., target-specific) matter to reduce the occurrence of side effects.

In addition to inducing an anti-viral interferon response, dsRNA also induces post-transcription gene silencing, a highly conserved anti-viral mechanism known as RNA interference (RNAi). Briefly, the RNA III Dicer enzyme processes dsRNA into short interfering RNA (siRNA) of approximately 22 nucleotides. The antisense strand of the siRNA binds a target mRNA via base pairing and serves as a guide sequence to induce cleavage of the target mRNA by an RNA-induced silencing complex RISC. dsRNA has been an extremely powerful tool in studying gene functions in C. elegens and Drosophila via gene silencing. However, its use in mammalian cells has been limited because the interferon response it elicits is detrimental to most mammalian cells.

Subsequently, it was found that siRNA was also capable of inducing RNAi, causing degradation of the target mRNA in a sequence-specific manner and it was thought to be short enough to bypass dsRNA-induced nonspecific effects in mammalian cells (Elbashri SM et al. 2001, Nature 411:494-498). Since then, siRNA has been widely used as a gene silencing tool in deciphering mammalian gene functions in research and drug discovery, and there has been great interest in its potential in therapeutic applications.

siRNA can be used to reduce or even abolish the expression of disease/disorder-related genes for preventing or treating diseases caused by the expression or overexpression of
the disease-related genes. Such diseases include, but are not limited to, infections, metabolic diseases, autoimmune diseases and cancer. However, concern has been raised recently about the potential for siRNA to activate immune responses which may be undesirable for certain indications and thus limit the use of siRNA as a gene silencing agent for therapeutic purposes (Sioud M et al. 2003, Biochem. Biophys. Res. Commun. 312:1220-1225). Therefore, there is a need for methods for predicting the potential of a given siRNA to induce an interferon response and for methods for designing and preparing siRNAs for gene silencing which are devoid of unwanted immunostimulatory activities.

On the other hand, for certain therapeutic applications, for example, the prevention or treatment of cancer and viral infections, immunostimulatory activity may be desirable as an additional functional activity of the siRNA.

In an effort to apply siRNA for the specific downregulation of TLR9 in PDC in our previous publication (Hornung V et al. 2005, Nat Med 11: 263-270), we made the surprising observation that, despite the inability of PDC to detect long dsRNA, certain siRNA sequences were potent in vitro inducers of IFN-α in PDC. We found that i) short interfering RNA (siRNA) induces IFN-α in human plasmacytoid dendritic cells when transfected with cationic lipids, ii) this activity of siRNA is sequence-dependent but independent of the G or U content of the siRNA, iii) the immunostimulatory activity of siRNA and the antisense activity are two independent functional activities of siRNA, iv) the immune recognition of siRNA occurs on the single strand level, v) siRNAs containing the 9mer sequence motif 5'-'GUCCUUCAA-3' show potent immunostimulatory activity, and vi) such siRNAs induce systemic immune responses in mice, and vii) the induction of immune responses by siRNA requires the presence of TLR7 in mice. Our findings suggest that the 9mer sequence motif 5’-GUCCUUCAA-3’ may be a ligand for TLR7.

The natural ligand for TLR7 has not been well defined to date. Guanine analogues have been identified earlier as specific ligands for TLR7 and TLR8 (Lee J et al. 2003, Proc Natl Acad Sci USA 100: 6646-6651; Heil F et al. 2003, Eur J Immunol 33: 2987-2997), whereas guanosine ribonucleoside or a derivative thereof has been identified as TLR7 ligand in WO03086280.
It is an object of the present invention to identify RNA oligonucleotide motifs for stimulating an immune response, in particular, IFN-α induction. It is also an object of the present invention to identify ligands for activating TLR7 and TLR8. It is another object of the present invention to develop a method for determining the immunostimulatory activity, in particular, the IFN-α-inducing activity, of a RNA oligonucleotide. It is yet another object of the present invention to develop a method for predicting the immunostimulatory activity, in particular, IFN-α-inducing activity, of a RNA oligonucleotide. It is a further object of the invention to develop a method for designing and preparing RNA oligonucleotide having or lacking immunostimulatory activity, in particular, IFN-α-inducing activity. It is also an object of the invention to provide RNA oligonucleotides having high immunostimulatory activity which can be used to induce an immune response, in particular, IFN-α production, in patients in need thereof. It is yet another object of the present invention to provide siRNA molecules that either have or lack immunostimulatory activity which can be used to treat disorders caused by the expression or overexpression of disorder-related genes.

**Summary of the Invention**

The present invention provides a method for determining the immunostimulatory activity of a RNA oligonucleotide, a method for predicting the immunostimulatory activity of a RNA oligonucleotide, a method for preparing a RNA oligonucleotide with high or low immunostimulatory activity, and a method for preparing a RNA oligonucleotide with gene silencing activity and with high or low immunostimulatory activity.

The present application also provides an in vitro method for inducing IFN-α production from a mammalian cell, and an in vitro method for activating a dendritic cell.

The present invention further provides a RNA oligonucleotide with immunostimulatory activity, a RNA oligonucleotide with gene silencing activity and with high or low immunostimulatory activity, and the therapeutic uses thereof.

In addition, the present invention provides a pharmaceutical composition comprising one or more of the RNA oligonucleotides of the invention.
Brief Description of the Figures

Figure 1: PBMC of three individual donors were isolated and stimulated with ssRNA oligonucleotides 9.2sense (5'-AGCUUAACCUGUCCUCAA-3') and 9.2antisense (5'-UUGAAGGACAGGUUAAGCU-3') that were complexed with either Lipofectamine, poly-L-arginine, poly-L-histidine or poly-L-lysine in duplicates. 24 hours after stimulation supernatants were harvested and IFN-α was assessed by ELISA. Data are presented as mean values ± SEM.

Figure 2: PBMC of three different healthy donors were isolated and stimulated with poly-L-arginine complexed ssRNA oligonucleotides in duplicates. 44 hours after stimulation IFN-α production was assessed in supernatant via ELISA. For all tested ssRNA oligonucleotides, the mean values of the measured duplicates were normalized to the positive control ssRNA oligonucleotide 9.2sense (5'-AGCUUAACCUGUCCUCAA) by dividing the mean value of tested oligonucleotide by the mean value of 9.2sense (=100%). Data from three different donors were summarized and are presented as mean values ± SEM.

Figure 3: PBMC of six different healthy donors were isolated and stimulated with poly-L-arginine complexed ssRNA oligonucleotides in duplicates. 44 hours after stimulation IFN-αa production was assessed in supernatant via ELISA. For all tested ssRNA oligonucleotides, the mean values of the measured duplicates were normalized to the positive control ssRNA oligonucleotide 9.2sense (5'-AGCUUAACCUGUCCUCAA) by dividing the mean value of tested oligonucleotide by the mean value of 9.2sense (= IFN-α index of a given oligonucleotide). Next, all individual IFN-α indices were adjusted to the mean value of all IFN-α indices by subtracting the mean value of all IFN-α indices from the individual IFN-α index of a given oligonucleotide (= adjusted IFN-α index). Data from six individual donors were summarized and were assorted in ascending order displaying the corresponding SEM. In addition, a statistical analysis was performed to assess a putative significant difference for the adjusted IFN-α indices of all top thirty ssRNA oligonucleotides. A two-tailed Student's t-test was employed to calculate the p-value off all possible ssRNA oligonucleotide combinations. A p-value > 0.01 and < 0.05 is depicted by a black box, whereas a p-value < 0.01 is depicted as a grey box.
Figure 4: The occurrence of 1mer motifs (5'-X-3'), 2mer motifs (5'-XX-3', 5'-X*X-3', 5'-X**X-3') and 3mer motifs (5'-XXX-3', 5'-XX*X-3', 5'-X**XX-3') in ssRNA oligonucleotides with an IFN-α index below the mean IFN-α index (group 1) or above the mean IFN-α index (group 2) was analyzed. The relative occurrence of a given motif within a group of ssRNA oligonucleotides was calculated by dividing the absolute number of occurrences of a given motif within a group through the absolute number of occurrences of all possible motifs within this group. A significant overrepresentation or underrepresentation of a given motif was analyzed using a chi-square test. The null hypothesis of equal distribution within both groups was rejected when the calculated p-value was below 0.05 (significant differences in distribution are indicated by "*"). For all motifs analyzed, relative occurrences are depicted in Figure 4 for group 1 oligonucleotides (black bars) and for group 2 oligonucleotides (white bars): 1mer motifs 5'-X-3' (Figure 4A); 2mer motifs 5'-XX-3' (Figure 4B1), 5'-X*X-3' Figure (Figure 4B2), 5'-X**X-3' (Figure 4B3) and 3mer motifs 5'-XXX-3' (Figure 4C1-4C4), 5'-XX*X-3' (Figure 4C5-4C8), 5'-X**XX-3' (Figure 4C9-4C12).

Figure 5: For all possible 1mer motifs (5'-X-3'), 2mer motifs (5'-XX-3', 5'-X*X-3', 5'-X**X-3') or 3mer motifs (5'-XXX-S', 5'-XX*X-3', 5'-X**XX-3') a mean IFN-α index was assigned by calculating a mean IFN-α index of all ssRNA oligonucleotides containing the corresponding motifs (= IFN-α score of a given motif). The IFN-α score of all possible motifs is depicted in Figure 5 ± SEM: 1mer motifs 5'-X-3' (Figure 5A); 2mer motifs 5'-XX-3' (Figure 5B1), 5'-X*X-3' Figure (Figure 5B2), 5'-X**X-3' (Figure 5B3) and 3mer motifs 5'-XXX-3' (Figure 5C1-5C4), 5'-XX*X-3' (Figure 5C5-5C8), 5'-X**XX-3' (Figure 5C9-5C12).

Figure 6: A calculated IFN-α index was assigned to each oligonucleotide by using the obtained motif-IFN-α scores. For each set of motifs [1mer motifs (5'-X-3'), 2mer motifs (5'-XX-3', 5'-X*X-3', 5'-X**X-3') or 3mer motifs (5'-XXX-3', 5'-XX*X-3', 5'-X**XX-3')] a predicted IFN-α index was calculated for each ssRNA oligonucleotide. Next, the obtained predicted IFN-α indices were compared to the actual adjusted IFN-α indices. Data are depicted the following way: For all ssRNA oligonucleotides the predicted IFN-α indices are shown as a black bars, whereas data are sorted in ascending order according to the actual IFN-α score that is depicted as a red index line. The y-axis on the left side depicts the scale for the predicted IFN-α score, while the y-axis on the right side depicts the scale for the actual IFN-α score.
Figure 7: PBMC from healthy donors were isolated and stimulated with poly-L-arginine complexed ssRNA oligonucleotides in duplicates. 44 hours after stimulation IFN-α production was assessed in supernatant via ELISA. For all tested ssRNA oligonucleotides, the mean values of the measured duplicates were normalized to the positive control ssRNA oligonucleotide 9.2sense (5'-AGCUUAACCUGUCCUUCAA) by dividing the mean value of tested oligonucleotide by the mean value of 9.2sense (= 1). A: A panel of ssRNA oligonucleotides was tested with different positions of the 5'-GUCA-3'-motif within the 19mer ssRNA oligonucleotide (see table 4). The 5'-GUCA-3'-motif is indicated by bold letters. Data from two independent donors were summarized and are depicted as mean values ± SEM. B/C: 16 ssRNA oligonucleotides, which included all possible oligonucleotides with permuted bases at the flanking positions to the 5'- and the 3'-end of the central 5'-GUCA-3'-motif (table 5), were complexed with poly-L-arginine and used to stimulate PBMC. 44 hours after stimulation IFN-α production was assessed in supernatant via ELISA. Data for all 16 oligonucleotides from three independent donors were summarized as mean values ± SEM (B). In addition all 16 oligonucleotides were assorted into groups according to the base preceding or following the central 5'-GUCA-3'-motif (C). On the left side oligonucleotides with a common base preceding the central 5'-GUCA-3'-motif were grouped, whereas on the right side oligonucleotides with a common base following the central 5'-GUCA-3'-motif were grouped. Individual ssRNA oligonucleotide IFN-α data were summarized according to the respective group and are depicted as mean values ± SEM. A two-tailed Student’s t-test was used to calculate a statistically significant difference between the various groups (p < 0.05 is indicated by a “*”).

Figure 8: A: PBMC from two healthy donors were isolated and stimulated with poly-L-arginine complexed ssRNA oligonucleotides (Table 6) in duplicates. 44 hours after stimulation IFN-α production was assessed in supernatant via ELISA. IFN-α data were summarized as mean values and subsequently normalized to the positive control RNA9.2sense (5'-AGCUUAACCUGUCCUUCAA-3'). In addition, respective sequences were analyzed using the IFN-α point score matrix (Table 7) and subsequently normalized to RNA9.2sense. A correlation coefficient of 0.84 was calculated for these two sets of data. Measured IFN-α levels are depicted in white bars, whereas predicted IFN-α scores are shown in black bars (A). Next, IFN-α point score matrix was employed to analyze IFN-α-inducing RNA oligonucleotides that have been described in the literature. Given
the fact that in the study performed by Judge et al. (2005, Nat Biotechnol 23:457-462) double-stranded RNA oligonucleotides were tested, a mean value for the individually analyzed single-stranded components was calculated. Data were normalized to the most potent RNA oligonucleotide (=100%) within the respective panel of oligonucleotides (B). For the prediction of single-stranded RNA oligonucleotides reported by Heil et al. (2004 Science 303:1526-1529), the predicted IFN-α point scores are depicted (C).

Figure 9: PBMC from individual healthy donors were isolated and stimulated with poly-L-arginine complexed ssRNA-oligonucleotides in duplicates. For a detailed list of all tested oligonucleotides see Table 1. 44 hours after stimulation IFN-α production was assessed in supernatant via ELISA. For all tested ssRNA-oligonucleotides, the mean values of the measured duplicates were normalized to the positive control ssRNA-oligonucleotide 9.2sense (5’-AGCUUAACCUGCCUUCAA) by dividing the mean value of tested oligonucleotide by the mean value of 9.2sense (= 100%). Data from nine different (A-D) donors or three different donors (E) were summarized and are presented as mean values ± SEM.

Figure 10: PBMC from individual healthy donors were isolated and stimulated with poly-L-arginine complexed ssRNA-oligonucleotides in duplicates. For a detailed list of all tested oligonucleotides see Table 13. 44 hours after stimulation IFN-α production was assessed in supernatant via ELISA. For all tested ssRNA-oligonucleotides, the mean values of the measured duplicates were normalized to the positive control ssRNA-oligonucleotide 9.2sense (5’-AGCUUAACCUGCCUUCAA) by dividing the mean value of tested oligonucleotide by the mean value of 9.2sense (= 100%). Data from two different donors were summarized and are presented as mean values ± SEM.

Figure 11: PBMC of six different healthy donors were isolated and stimulated with poly-L-arginine complexed ssRNA-oligonucleotides in duplicates. 44 hours after stimulation IFN-α production was assessed in supernatant via ELISA. For all tested ssRNA-oligonucleotides, the mean values of the measured duplicates were normalized to the positive control ssRNA-oligonucleotide 9.2sense (5’-AGCUUAACCUGCCUUCAA) by dividing the mean value of tested oligonucleotide by the mean value of 9.2sense. Next, for each individual donor a global normalization to the mean was performed by subtracting the mean of all data from a particular donor from the individual raw data. Data from six individual donors were visualized using tree view and are depicted in
ascending order (A). In addition all individual data were summarized as mean values ±SEM and are depicted in ascending order (B).

Figure 12: The occurrence of 3mer motifs in all ssRNA-oligonucleotides was analyzed. The mean level of IFN-α induction was calculated by grouping all oligonucleotides that contained a respective 3mer motif. For example the 3mer motif 5′-GUC-3′ was contained in ssRNA oligonucleotides ANP 35, 83, 131, 137, 138, 139 and 179 with respective IFN-α induction levels of 1.33, 0.68, 0.93, 0.79, 0.44, 0.84 and 0.73. The mean IFN-α induction level of the 3mer motif 5′-GUC-3′ was thus calculated to be 0.82 with a standard error of mean of 0.10. 3mer motifs that were gapped by one nucleotide between either the first and the second nucleotide position (5′-N-NN-3′) or the second and third nucleotide position (5′-NN-N-3′) were also included in the analysis. A two-tailed T-Test was used to identify motifs that were either significantly higher or lower in IFN-α induction than the residual motifs. For all motifs analyzed, the mean IFN-α induction level was visualized using tree view. The data were assorted according to the first nucleotide position of the motif in four groups, (p-value < 0.05 is indicated by *).

Figure 13: The top 15 percent of all ssRNA oligonucleotides and the respective mean IFN-α induction levels are shown in ascending order. The presence of the identified potent 3mer motifs 5GUY-3′ (5′-GUC-3′ 5′-GUU-3′), 5′-GUNY-3′ (5′-GUNC-3′ 5′-GUNU-3′) and 5′-GNYU-3′ (5′-GNUC-3′ 5′-GNUU-3′) is indicated by a grey box. All ssRNA oligonucleotides that contain any of the above motifs are indicated by a black box.

Figure 14: PBMC of four different healthy donors were isolated and stimulated with the following poly-L-arginine complexed ssRNA-oligonucleotides: ANP143 (5′-AAAAAAAGU UCAAAAAAAA-3′), RNA40 (5′-GCCGUCUGUUGUGAGACUC-3′), β-Gal control sense (5′-UUGAUGUGUUAGUCGAU-3′) and 9.2sense (5′-AGCUUAACCUGCUUCAA-3′). 44 hours after stimulation IFN-α production was assessed in supernatant via ELISA. All tested ssRNA-oligonucleotides were normalized to the positive control ssRNA-oligonucleotide 9.2sense by dividing the mean value of tested oligonucleotide by the mean value of 9.2sense. Significant differences were analyzed using a two-tailed T-Test.

Figure 15: The occurrence of 3mer motifs was analyzed in the 193 oligonucleotide library. For each oligonucleotide the 3mer motif with the highest calculated mean IFN-α
induction level was identified and assigned to the respective oligonucleotide. The predicted data are depicted in ascending order (black bars) according to the corresponding measured IFN-α induction levels (black line). In addition, the correlation coefficient for the two data sets was determined.

Figure 16: The occurrence of 3mer motifs was analyzed in the 193 oligonucleotide library. For each oligonucleotide the 3mer motif with the highest calculated mean IFN-α induction level was identified and assigned to the respective oligonucleotide. The predicted data are depicted in ascending order. Various threshold levels (dotted lines) were tested for both for the positive predictive value and the sensitivity to identify oligonucleotides below the IFN-α induction level of 0 (A). For each threshold level, data were then regrouped according to the predicted IFN-α induction level (selected oligonucleotides: left group, eliminated oligonucleotides: right group). For each group, the mean level of IFN-α induction ± SEM is depicted in the lower panel. The positive predictive value and the sensitivity for each threshold is indicated in the upper left.

Figure 17: The prediction algorithm was used to analyze all possible siRNA duplexes targeting the mRNA of human TLR9 (NM_017442). For the 3868 bp long mRNA of TLR9 all possible 19mer siRNA duplexes were considered and the IFN-α prediction algorithm was applied on both the sense and the antisense strand of each siRNA duplex. The predicted IFN-α induction levels are depicted in stacked columns for the sense (upper columns in black) and the antisense strand (lower column in grey). The relative targeting position of the siRNA duplex is given on the y-axis, whereas the predicted IFN-α induction is depicted on the x-axis (A). In addition, six selected regions of the TLR9 mRNA and the respective predicted IFN-α induction levels are depicted in detail in B.

Figure 18: HEK 293 cells were transfected with an expression plasmid coding for human TLR9 with a C-terminal YFP-tag. Various siRNA-duplexes targeting human TLR9 mRNA were cotransfected. The starting base of the individual siRNA is given in the lower panel. 20 hours after transfection, TLR9 expression was analyzed by flow cytometry. Data are depicted as percentage of TLR9-expression referring to an irrelevant control siRNA as 100% and siRNA_sb1647 as 0%. Results are shown as mean values ± SEM (n=3) (A). In addition, above siRNA duplexes and the respective single stranded components were used to transfect human PBMC from five individual donors. 40 hours
after transfection IFN-α induction was measured via ELISA. Data are depicted as mean values ±SEM (B).

Figure 19: Based on the algorithm described in example 17, a computer program was written that applies the algorithm to all possible siRNA duplexes targeting all human RNA transcripts (50421 as of 09/2006) as published by the National Center for Biotechnology Information (NCBI). Each entry into the NCBI database (ftp://ftp.ncbi.nih.gov/refseq/H_sapiens/mRNA_Prot/human.ma.fna.gz) of all listed human RNA transcripts was analyzed the following way: A list of all possible 19mer siRNA duplexes targeting a given RNA transcript was generated. Of all siRNA duplexes the IFN-α induction of both the sense and the antisense strand was predicted using the method described in example 17. The obtained data is stored in a database (CD-ROM) and can be retrieved by a search engine. Using the search interface, the user can pick the transcript of interest (alphabetical index of all RNA transcripts targeted by siRNAs) and then adjust the level of threshold to identify siRNA duplexes that are of either low, intermediate or high in immunostimulatory activity (A). For example, using the threshold of 0.11 as described in example 17, a set of siRNA duplexes was identified for Homo sapiens vascular endothelial growth factor (VEGF) transcript variant 1 mRNA (NM_001025366.1) with low immunostimulatory activity for both the sense and the antisense strand (B).

Detailed Description of the Invention

Definitions

As used herein, "a" and "an" refers to a group or species of entities, rather than one single individual.

oligonucleotide

As used herein, the term "oligonucleotide" refers to a polynucleotide formed from a plurality of linked nucleoside units. Such oligonucleotides can be obtained from existing nucleic acid sources, including genomic or cDNA, but are preferably produced by synthetic methods including chemical synthesis, in vitro and in vivo transcription. In
preferred embodiments each nucleoside unit includes a heterocyclic base and a pentofuranosyl, trehalose, arabinose, 2'-deoxy-2'-substituted arabinose, 2'-0-substituted arabinose or hexose sugar group. The nucleoside residues can be coupled to each other by any of the numerous known internucleoside linkages. The term "oligonucleotide" also encompasses polynucleosides having one or more stereospecific internucleoside linkage (e.g., (R)_p, (S)_p, phosphotriester, alkylphosphonate, alkylphosphonothioate, phosphotriester, phosphoramidate, siloxane, carbonate, carboalkoxy, acetamide, carbamate, morpholino, borano, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate, and sulfone internucleoside linkages). The modified nucleosides of the invention can include naturally occurring nucleosides, modified nucleosides, or mixtures thereof. As used herein, the term "modified nucleoside" is a nucleoside that includes a modified heterocyclic base, a modified sugar moiety, or a combination thereof. In some embodiments, the modified nucleoside is a non-natural pyrimidine or purine nucleoside, as herein described. In some embodiments, the modified nucleoside is a 2'-substituted ribonucleoside an arabinonucleoside or a 2'-deoxy-2'-substituted-arabinoside.

As used herein, the term "2'-substituted ribonucleoside" or "2'-substituted arabinoside" includes ribonucleosides or arabinonucleoside in which the hydroxyl group at the 2' position of the pentose moiety is substituted to produce a 2'-substituted or 2'-O-substituted ribonucleoside. Preferably, such substitution is with a lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an aryl group having 6-10 carbon atoms, wherein such alkyl, or aryl group may be unsubstituted or may be substituted, e.g., with halo, hydroxy, thfluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carboalkoxy, or amino groups. Examples of 2'-0-substituted ribonucleosides or 2'-O-substituted-arabinosides include, without limitation 2'-O-methylribonucleosides or 2'-O-methylarabinosides and 2'-O-methoxyethylribonucleosides or 2'-O-methoxyethylarabinosides.

The term "2'-substituted ribonucleoside" or "2'-substituted arabinoside" also includes ribonucleosides or arabinonucleosides in which the 2'-hydroxyl group is replaced with a lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an
amino or halo group. Examples of such 2'-substituted ribonucleosides or 2'-substituted arabinosides include, without limitation, 2'-amino, 2'-fluoro, 2'-allyl, and 2'-propargyl ribonucleosides or arabinosides.

The term "oligonucleotide" includes hybrid and chimeric oligonucleotides. A "chimeric oligonucleotide" is an oligonucleotide having more than one type of internucleoside linkage. One preferred example of such a chimeric oligonucleotide is a chimeric oligonucleotide comprising a phosphorothioate, phosphodiester or phosphorodithioate region and non-ionic linkages such as alkylphosphonate or alkylphosphonothioate linkages (see e.g., Pederson et al. U.S. Pat. Nos. 5,635,377 and 5,366,878).

A "hybrid oligonucleotide" is an oligonucleotide having more than one type of nucleoside. One preferred example of such a hybrid oligonucleotide comprises a ribonucleotide or 2'-substituted ribonucleotide region, and a deoxyribonucleotide region (see, e.g., Metelev and Agrawal, U.S. Pat. Nos. 5,652,355, 6,346,614 and 6,143,881).

RNA oligonucleotides discussed herein include otherwise unmodified RNA as well as RNA which have been modified (e.g., to improve efficacy), and polymers of nucleoside surrogates. Unmodified RNA refers to a molecule in which the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are the same or essentially the same as that which occur in nature, preferably as occur naturally in the human body. The art has referred to rare or unusual, but naturally occurring, RNAs as modified RNAs, see, e.g., Limbach et al. 1994, Nucleic Acids Res 22: 2183-2196. Such rare or unusual RNAs, often termed modified RNAs (apparently because these are typically the result of a post-transcriptional modification) are within the term unmodified RNA, as used herein. Modified RNA as used herein refers to a molecule in which one or more of the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are different from that which occurs in nature, preferably different from that which occurs in the human body. While they are referred to as modified "RNAs," they will of course, because of the modification, include molecules which are not RNAs. Nucleoside surrogates are molecules in which the ribophosphate backbone is replaced with a non-ribophosphate construct that allows the bases to the presented in the correct spatial relationship such that hybridization is substantially similar to what is seen with a ribophosphate backbone, e.g., non-charged mimics of the ribophosphate backbone.
All nucleic acid sequences listed herein are in the 5' to 3' direction unless otherwise indicated.

The RNA oligonucleotide of the invention can be single-stranded, double stranded, or partially double-stranded.

A single-stranded RNA oligonucleotide may contain self-complementary sequences and forms a hairpin. For example, 5'-GACCUAGCCUAAAACUAGGUC-3'. The self-complementary sequence may be a palindromic sequence. For example, 5'AAAGAUAUCCGAUCAAAA-3'.

A double stranded RNA oligonucleotide may have one- or two-nucleotide overhang at the 5' or 3' end of one or both strands.

A partially double-stranded RNA oligonucleotide may comprise two strands of the same or different length, wherein the at least one of the strands contains nucleotides outside the complementary sequence. For example,

Example 1: 5'-AAAAGUUCAAAAGCUCAAAA-3'
            3'-CAAGUUUCGAG-5'

Example 2: 5'-UCAAAGUCAAAAAGCUCAAGAUUGAAAGUUAAA-3'
            3'-GACUUGAAAAUUUCAGUUUUCGAGUUUAAGUUGAAAAACUCG-5'

Example 3: 5'-UCAAAGUCAAAAAGCUCAAGAUUGAAA-3'
            3'-UUUCAGUUUUCGAGUUUAAGUUGAAAAACUCG-5'

The length of a single-stranded RNA oligonucleotide is the number of nucleotides contained in the oligonucleotide.

In the case of a double-stranded or partially double-stranded oligonucleotide, the length of the oligonucleotide is the length of the individual strands. In other words, a partially double-stranded oligonucleotide can have two lengths.

Enhanced Nuclease Resistance
For increased nuclease resistance and/or binding affinity to the target, an oligonucleotide can include, for example, 2'-modified ribose units and/or phosphorothioate linkages and/or pyrophosphate linkages. For example, the 2' hydroxyl group (OH) can be modified or replaced with a number of different "oxy" or "deoxy" substituents.

Examples of "oxy"-2' hydroxyl group modifications include alkoxy or aryloxy (OR, e.g., R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG), 0(CH₂CH₂O)ₙCH₂CH₂OR; "locked" nucleic acids (LNA) in which the 2' hydroxyl is connected, e.g., by a methylene bridge, to the 4' carbon of the same ribose sugar; O-AMINE and aminoalkoxy, O(CH₂)ₙAMINE, (e.g., AMINE = NH₂; alkylamino, dialkylamino, heterocyclyl amino, arylamino, diaryl amino, heteroarylamino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides containing only the methoxyethyl group (MOE), (OCH₂CH₂OCH₃, a PEG derivative), exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

"Deoxy" modifications include hydrogen (i.e. deoxyribose sugars, which are of particular relevance to the overhang portions of partially ds RNA); halo (e.g., fluoro); amino (e.g. NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); NH(CH₂CH₂NH)ₙCH₂CH₂-AMINE (AMINE = NH₂; alkylamino, dialkylamino, heterocyclyl amino, arylamino, diaryl amino, heteroarylamino, or diheteroarylamino), -NHC(O)R (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with e.g., an amino functionality.

Preferred substituents are 2'-methoxyethyl, 2'-OCH₃, 2'-O-allyl, 2'-C- allyl, and 2'-fluoro. To maximize nuclease resistance, the 2' modifications can be used in combination with one or more phosphate linker modifications (e.g., phosphorothioate). The so-called "chimeric" oligonucleotides are those that contain two or more different modifications.

The inclusion of furanose sugars in the oligonucleotide backbone can also decrease endonucleolytic cleavage. An oligonucleotide agent can be further modified by including a 3' cationic group, or by inverting the nucleoside at the 3'-terminus with a 3'-3' linkage. In another alternative, the 3'-terminus can be blocked with an aminoalkyl group, e.g., a 3' C5-aminoalkyl dT. Other 3' conjugates can inhibit 3'-5' exonucleolytic cleavage. While not being bound by theory, a 3' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 3'-end.
of oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

Similarly, 5'-conjugates can inhibit 5'-3' exonucleolytic cleavage. While not being bound by theory, a 5' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 5'-end of oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

Single-stranded RNA oligonucleotides which contain self-complementary sequences and form a hairpin structure have enhanced nuclease resistance compared to single-stranded oligonucleotides which do not.

5'-Phosphate Modifications

The oligonucleotides of the present invention can be 5' phosphorylated or can include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications of the antisense strand include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)P-O-5'); 5'-diphosphate ((HO)P-O-P-HO-O-5'); 5'-triphosphate ((HO)P-O-(HO)P-O-P-HO-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'(HO)P-O-(HO)P-O-P-HO-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure. Other suitable 5'-phosphate modifications will be known to the skilled person.

Tethered Ligands

The RNA oligonucleotides of the present invention also include those with tethered ligands. The properties of a RNA oligonucleotide, including its pharmacological properties, can be influenced and tailored by the introduction of ligands, e.g. tethered ligands.
The ligands may be coupled, preferably covalently, either directly or indirectly via an intervening tether, to the RNA oligonucleotide. In preferred embodiments, the ligand is attached to the oligonucleotide via an intervening tether.

In preferred embodiments, a ligand alters the distribution, targeting or lifetime of a RNA oligonucleotide into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, e.g., molecule, cell or cell type, a cellular or organ compartment, tissue, organ or region of the body.

Preferred ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified oligoribonucleotide, or a polymeric molecule comprising any combination of monomers described herein and/or natural or modified ribonucleotides.

A wide variety of ligands may be used. Ligands may include agents that allow for the specific targeting of the oligonucleotide; diagnostic compounds or reporter groups which allow for the monitoring of oligonucleotide distribution; cross-linking agents; nuclease-resistance conferring moieties; and natural or unusual nucleobases. General examples include lipophilic moleculenes, lipids, lectins, steroids (e.g., uvaol, hecigenin, diosgenin), terpenes (e.g., triterpenes, e.g., sarsasapogenin, Friedelin, epifriedelanol derivatized lithocholic acid), vitamins, carbohydrates (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid), proteins, protein binding agents, integrin targeting molecules, polycationics, peptides, polyamines, and peptide mimics.

The ligand may be a naturally occurring or recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid. Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic moieties, e.g., cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.
Ligands can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a thyrotropin, melanotropin, surfactant protein A, Mucin carbohydrate, a glycosylated polyaminoacid, transferrin, bisphosphonate, polyglutamate, polyaspartate, or an RGD peptide or RGD peptide mimetic.

Ligands can be proteins, e.g., glycoproteins, lipoproteins, e.g. low density lipoprotein (LDL), or albumins, e.g. human serum albumin (HSA), or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-glucosamine, multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB. The ligand can be a substance, e.g., a drug, which can increase the uptake of the oligonucleotide agent into the cell, for example, by disrupting the cell's cytoskeleton, e.g., by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

In one embodiment, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, e.g., human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, e.g., liver tissue, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, e.g., HSA.

A lipid based ligand can be used to modulate, e.g., control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In another embodiment, the ligand is a moiety, e.g., a vitamin or nutrient, which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating
disorders characterized by unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include the B vitamins, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells.

In another embodiment, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennapedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

In a preferred embodiment, the ligand is an antibody or a fragment thereof which is specific for a moiety present in a cell to be targeted. The moiety may be a protein, a carbohydrate structure, a polynucleotide, or a combination thereof. The moiety may be secreted, associated with the plasma membrane (e.g., on the extracellular or intracellular surface), cytosolic, associated with intracellular organelles (e.g., ER, Golgi complex, mitochondria, endosome, lysosome, secretory vesicle) or nuclear. The antibody may be monoclonal or polyclonal. The antibody may be chemeric or humanized. The antibody may be a single chain antibody. The antibody fragment may be a Fab fragment, a F(ab')2 fragment, or any fragments that retain the antigen-binding specificity of the intact antibody.

**immunostimulatory activity**

As used herein, "immunostimulatory activity" refers to the capability of a molecule or a composition to induce an immune response. In one aspect, the immunostimulatory activity refers to the type I-IFN-inducing activity, in particular, the IFN-α-inducing activity.

As used herein, "inducing an immune response" means initiating or causing an increase in one or more of B-cell activation, T-cell activation, natural killer cell activation, activation of antigen presenting cells (e.g., B cells, dendritic cells, monocytes and macrophages), cytokine production, chemokine production, specific cell surface marker expression, in
particular, expression of co-stimulatory molecules. In one aspect, such an immune response involves the production of type I IFN, in particular, IFN-α, in cells such as PDC.

As used herein, "IFN-α-inducing activity" refers to the capability of a molecule or composition to induce IFN-α production from a cell capable of producing IFN-α. Cells capable of producing IFN-α include, but are not limited to, peripheral blood mononuclear cells (PBMC) (e.g., B cells, dendritic cells (myeloid dendritic cells and plasmacytoid dendritic cells), macrophages, monocytes, natural killer cells, granulocytes), endothelial cells, and cell lines (e.g., THP1; cells transfected with expression vectors for TLR-7 and/or TLR-8 such as CHO cells, COS cells, HEK293 cells). Cells capable of producing IFN-α include those that express TLR7, TLR8, or both TLR7 and TLR8.

gene silencing activity

As used herein, "gene silencing" refers to the downregulation or the abolition of the expression of a target gene. Gene silencing as used herein, occurs at the post-transcriptional level. Gene silencing may be directly or indirectly mediated by siRNA, shRNA and antisense RNA.

Both the antisense-strand of the siRNA and the antisense RNA have complementary to the target mRNA and are the effector strand of the gene silencing activity. The term complementary is well understood by those skilled in the art. For example, A is complementary to T, G is complementary to C, 5'-AG-3' is complementary to 5'-CT-3'.

The degree of complementarity between two oligonucleotides is the percentage of complementary bases in the overlapping region of the two oligonucleotides. The degree of complementarily can be determined manually or automatically by various engines such as BLAST. For example, ATCG has 100% complementarity to CGAT and CGATGG, and 75% complementarity to CGTT and CGTTGG. Furthermore, the degree of complementarity between a RNA oligonucleotide and any sequences present in the public databases (e.g., EMBL, GeneBank) can be determined by the BLAST program.

The degree of complementarity between the antisense strand of the siRNA or the antisense RNA and the target mRNA is at least 80% 81%, 82%, 83%, preferably at least
84%, 85%, 86%, 87%, 88%, more preferably at least 89%, 90%, 91%, 92%, 93%, even more preferably at least 94%, 95%, 96%, 97%, 98%, 99%, and most preferably 100%.

The gene silencing activity of a RNA oligonucleotide can be determined experimentally by methods well known in the art. For Example, the RNA oligonucleotide may be introduced into a cell by a method known in the art such as transfection and transduction; the mRNA level of the target gene can be determined by routine methods such as Northern blot analysis, quantitative PCR, RNase protection assay, and branching DNA; and the protein expression level can be determined by routine methods such as Western blotting, ELISA, and biological activity assays specific to the target protein. Furthermore, the mRNA level of all known and hypothetical genes can be determined at the global level using the microarray technology. Technologies in the field of proteomics allow for the protein levels of a large number of genes to be determined at the global level as well.

Naked RNA oligonucleotide may be transfected into a cell via electroporation. RNA oligonucleotide may be complexed with a complexation agent which facilitates the uptake of the oligonucleotide into a cell. Such complexation agents include, but are not limited to cationic lipids (e.g., Lipofectamine, Oligofectamine, DOTAP), cationic peptides, and calcium phosphate.

The gene silencing activity of a RNA oligonucleotide can be predicted by algorithms such as the one disclosed in Reynolds et al. 2004, Nat Biotechnol 22:326-330.

siRNA

As used herein, "siRNA" stands for short interfering RNA, and has the same definition as that established in the art. siRNA is double-stranded and is usually between 19 and 27 nucleotide in length. In vivo, siRNA is the product of Dicer activity on long dsRNA. The antisense strand of siRNA is complementary to the target mRNA; it binds the target mRNA and induces RISC-mediated target mRNA degradation. siRNA can be chemically synthesized, produced in vitro by Dicer-mediated enzymatic degradation of long dsRNA, produced by in vitro transcription from linear (e.g. PCR products) or circular templates (e.g., viral or non-viral vectors), or produced by in vivo transcription from viral or non-viral vectors. Commercially available synthetic siRNA usually contain a core of 19 complementary base pairs and a 2-nucleotide (UU or TT) 3' overhang on each strand.
siRNA may be chemically modified to have enhanced stability in vitro (especially in serum-containing media) and in vivo. siRNA may also be chemically modified to have enhanced uptake by cells in vitro and in vivo. Furthermore, siRNA may be linked to tethered ligands to have enhanced target specificity and improved pharmacological properties (such as half-life, clearance, distribution).

shRNA

As used herein, "shRNA" stands for short hairpin RNA and has the same definition as that established in the art. shRNA is processed inside a cell into siRNA which mediates RNAi as described previously. The loop sequence in shRNA is not thought to be involved in RNAi, and it can be of various lengths and sequences. The preferred lengths and sequences of the loop are known to those skilled in the art.

Similar to siRNA, shRNA can be chemically synthesized, produced by in vitro transcription from linear (e.g. PCR products) or circular templates (e.g., viral or non-viral vectors), or produced by in vivo transcription from viral or non-viral vectors.

antisense RNA

As used herein, "antisense RNA" has the same definition as that established in the art. Antisense RNA is complementary to target mRNA and it thought to interfere with the translation of the target mRNA. Antisense RNA molecules are usually 18-50 nucleotides in length. Antisense RNA may be modified to have enhanced stability, nuclease resistance, target specificity and improved pharmacological properties.

Similar to siRNA and shRNA, antisense RNA can be chemically synthesized, produced by in vitro transcription from linear (e.g. PCR products) or circular templates (e.g., viral or non-viral vectors), or produced by in vivo transcription from viral or non-viral vectors.

disorder/disease-related gene and antigen
As used herein, "disorder/disease-related gene" refers to a gene that is expressed or overexpressed in a disease/disorder and that is not expressed or expressed in reduced amount under normal condition. For example, a mutant CF gene is expressed in cystic fibrosis patient but not in an individual without cystic fibrosis; ErbB2 (or Her2) is overexpressed in breast cancer cells compared to normal breast cells; a viral gene is expressed in infected cells but not in uninfected cells. The gene product of the disorder/disease-related gene is referred to herein as the "disorder/disease-related antigen".

**mammal**

As used herein, the term "mammal" includes, without limitation, rats, mice, cats, dogs, horses, sheep, cattle, cows, pigs, rabbits, non-human primates, and humans.

**Technology Platform**

The vertebrate immune system established different ways to detect invading pathogens based on certain characteristics of their microbial nucleic acids. Detection of microbial nucleic acids alerts the immune system leading to the appropriate type of immune responses that is required for the defence against the respective type of pathogen detected. Detection of viral nucleic acids leads to the production of type I IFN, the key cytokine for anti-viral defence. While it is well established that the recognition of microbial DNA is sequence-specific, involving the so-called CpG motifs, the optimal motif for the recognition of microbial RNA has not been defined yet. The present application provides a technology platform for identifying the optimal motif for the recognition of microbial RNA and the induction of type I IFN.

The technology platform of the present invention comprises three key features: i) the transfection of peripheral blood mononuclear cells (PBMC) from healthy donors with RNA oligonucleotides; ii) the generation of a RNA oligonucleotide library containing 4mer motifs on a poly adenosine (polyA) backbone; iii) the development of algorithms based on the experimental data generated for the RNA oligonucleotide library to predict the immunostimulatory activity of any given RNA oligonucleotide.
The first key feature of the technology platform is the method of introducing RNA oligonucleotides into PBMC. Naked RNA oligonucleotides are not taken up by the cells to any significant degree. RNA oligonucleotides normally need to form complexes with complexation agent (or transfection agent) in order to be introduced into cells. In the literature, cationic lipids such as lipofectamine or DOTAP are routinely used as complexation agent for the transfection of RNA oligonucleotides. However, RNA-cationic lipid complexes lead to rapid cell death of myeloid cells. Although myeloid cells are not the cellular source of IFN-α within PBMC (the source is PDC), death of myeloid cells in the cell culture negatively affects the reproducibility of IFN-α induction in PBMC. Therefore, the use of cationic lipids is limited to isolated PDC. However, isolated PDC are not suitable for large scale screening assays because PDC make up 0.2-0.6% of the PBMC in a normal individual; it is difficult to obtain enough cells for the assays.

To identify a complexation agent that is suitable for use with PBMC, we compared different types of cationic peptides, poly-His, poly-L-Lys, and poly-L-Arg. Poly-L-Arg was found to provide the most potent support for the immunostimulatory activity of RNA oligonucleotides when compared to other cationic peptides and cationic lipids (Figure 1). A protocol was then established that allows well-controlled and highly reproducible complex formation between the RNA oligonucleotide and the complexation agent and subsequent RNA transfection into cells. Complex formation could be controlled by salt concentration, phosphate content and incubation time. Complex formation was monitored by the size of complexes and the functional activity over a range of concentrations. The use of poly-L-Arg did not affect the viability of myeloid cells and thus could be applied to PBMC without restrictions.

The second key issue of the technology platform was the generation of the RNA oligonucleotide library. An earlier study showed that a minimal length of 19 bases was required for the optimal immunostimulatory activity of an RNA oligonucleotide; furthermore, it showed that poly adenosine (poly A) was completely inactive (Hornung V et al. 2005, Nat Med 11:263-270). Therefore, the motif search was performed with a 19mer oligonucleotide on a poly A sequence background. By adding increasing numbers of uridine (U) in the center of such a poly A oligonucleotide, we found that a 4-nucleotide (4mer) motif in the center was sufficient to confer marked immunostimulatory activity (Figure 2). Importantly, after identifying the optimal 4mer sequence motifs for inducing IFN-α production, we found that changing the bases flanking the 4mer motifs did not
further enhance the immunostimulatory activity of the 4mer motifs (Figure 7B). The library of 193 RNA oligonucleotides used covered all 256 possible 4mer motifs. The reduction from 258 to 193 was possible because of redundant motifs caused by the poly A flanking regions. In additional studies we found that the exact location of the 4mer motif within the poly A backbone is not critical for the immunostimulatory activity (Figure 7A).

The third key feature of the technology platform was the generation of a data matrix and its mathematical analysis. Algorithms were developed that allowed an excellent prediction of the immunostimulatory activity of RNA oligonucleotides. The frequency of a given 4mer motif at a certain position within an oligonucleotide is only 1:256. Even though the most active 4mer motifs can be used as the core for constructing potent immunostimulatory RNA oligonucleotides, the IFN-α indices of the 4mer motifs are not particularly useful for predicting the activity of a given RNA oligonucleotide, or for designing RNA oligonucleotides with minimal immunostimulatory activity which is desired for an siRNA. Therefore, algorithms were established which based on parts of the 4mer motifs, namely 1, 2 or 3 bases either in a row (XXX) or with spacing (X*XX; XX*X). The highest predictive value was obtained with the algorithm using 3 bases (i.e., 3mer motifs). This 3mer-based algorithm allowed an impressively accurate prediction (correlation coefficient (r)=0.87) of the immunostimulatory activity of the 19mer RNA oligonucleotides carrying 4mer motifs in our library (Figure 6E) and RNA oligonucleotides previously published in the literature by us and others (Figure 8A-C).

There are a number of applications for the information generated by our technology platform: a) the 4mer motif data matrix can be used to design oligonucleotides with optimal IFN-α-inducing activity; b) 4mer motifs with minimal IFN-α-inducing activity can be used as the repertoire for selecting potential inhibitory sequence motifs; c) the 3mer-based algorithm (e.g., the IFN-α point score matrix) can be used to predict the immunostimulatory activity of a given RNA oligonucleotide; d) the 3mer-based algorithm (e.g., the IFN-α point score matrix) can be used to design RNA oligonucleotides with maximal immunostimulatory activity and additional sequence requirements for other functionalities such as gene silencing in the case of an siRNA (in this case the use of 4mer motif matrix is not useful since 4mer motifs are not frequent enough); e) the 3mer-based algorithm (e.g., the IFN-α point score matrix) can also be used to design RNA nucleotides with minimal immunostimulatory activity and additional sequence
requirements for other functionalities such as gene silencing in the case of an siRNA (in this case the use of 4mer motif matrix is not useful since 4mer motifs are not frequent enough).

In the case of an immunostimulatory RNA, an oligonucleotide containing only one of the most potent 4mer motifs is 80% more active than the most active complex oligonucleotide containing a 9mer motif in the literature (Table 1). In a 19mer oligonucleotide, there is room for several potent 4mer motifs. A 19mer RNA oligonucleotide containing more than one potent immunostimulatory 4mer motifs is expected to have even higher activity.

Furthermore, inhibitory motifs may exist that inhibit the immunostimulatory activity of a RNA oligonucleotide as in the case of CpG oligonucleotides. Such inhibitory motifs, by definition, are among the motifs with weak IFN-α-inducing activity. In the field of RNA interference, type I IFN induction usually is unwanted. The 3mer-based algorithm (e.g., the IFN-α point score matrix) described above can be used to select siRNA sequences with minimal immunostimulatory activity. A sequence analysis of cyclophylin B mRNA, one of the best studied targets for siRNA, identifies a number of siRNA sequences for which our algorithm (e.g., the IFN-α point score matrix) predicts minimal type I IFN induction and which still are known to be potent in gene silencing (Reynolds A et al. 2004, Nat Biotechnol 22: 326-330). This confirms our previous finding that RNA interference and IFN-α induction are two independent functional activities of a siRNA molecule.

Of note, the motif search performed in the present study focuses on the activity of RNA oligonucleotides to induce IFN-α. From previous studies, it is known that the cellular source of IFN-α within the PBMC is PDC. By analysing the level of IFN-α induction in PBMC, other activities of the RNA oligonucleotides on other cellular subsets of the PBMC, such as myeloid cells, are not addressed. Myeloid cells express TLR8 in addition to TLR7 and thus may show different nucleotide sequence specificities and may be induced to exhibit additional activities than IFN-α production. It therefore needs to be born in mind that ssRNA oligonucleotides are capable of inducing both PDC-dependent (i.e. IFN-α production) and PDC-independent activities (e.g., activation of myeloid cells). In contrast, we found that dsRNA oligonucleotides, such as siRNA, are only recognized
by PDC but not myeloid cells. As a result, it is valid to predict the immunological activity of siRNA oligonucleotides based on their ability to induce IFN-α production.

**Method for Determining the Immunostimulatory Activity of an RNA Oligonucleotide**

The present invention provides a method for determining the immunostimulatory activity, in particular, the IFN-α-inducing activity, of a RNA oligonucleotide, comprising the steps of:

(a) complexing the RNA oligonucleotide with a complexation agent;
(b) contacting a cell with the complexed RNA oligonucleotide, wherein the cell expresses TLR7 or TLR8 or both TLR7 and TLR8; and
(c) determining the amount of IFN-α produced by the cell of step (b), an increase of IFN-α production indicating immunostimulatory activity of the RNA oligonucleotide.

In one embodiment of the invention, the complexation agent is a polycationic peptide, preferably poly-L-arginine (poly-L-Arg). In one embodiment, the polycationic peptide, in particular, poly-L-Arg, is at least 24 amino acids in length. The polycationic peptide, in particular, poly-L-Arg, may be a heterogeneous mixture of peptides of different lengths.

The cells expressing TLR7 or TLR8 or both TLR7 or TLR8 include, but are not limited to, peripheral blood mononuclear cells (PBMC), plasmacytoid dendritic cells (PDC), myeloid dendritic cells (MDC), B cells, macrophages, monocytes, natural killer cells, granulocytes, endothelial cells, cell lines such as THP1, and cells containing exogenous DNA which directs the expression of TLR7 or TLR8 or both TLR7 or TLR8 such as transfected CHO, HEK293, and COS cells.

In one embodiment of the invention, the cell is a mammalian cell, preferably a human cell or a cell of human origin.

The RNA oligonucleotide can be single-stranded, double-stranded or partially double-stranded.
Method for Predicting the Immunostimulatory Activity of a RNA Oligonucleotide

The present invention provides a method for predicting the immunostimulatory activity, in particular the IFN-α-inducing activity, of a RNA oligonucleotide, comprising the steps of:

(a) identifying all possible 3-nucleotide (3mer) motifs contained in the oligonucleotide;

(b) assigning an IFN-α point score for each individual 3mer motif;

(c) assigning the sum of the IFN-α point scores of individual 3mer motifs as the IFN-α score of the oligonucleotide; and

(d) assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least 23, an intermediate immunostimulatory activity if the IFN-α score is between -4 and 23, and a low immunostimulatory activity if the IFN-α score is at most -4, when n=6;

assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least 26, an intermediate immunostimulatory activity if the IFN-α score is between -4 and 26, and a low immunostimulatory activity if the IFN-α score is at most -4, when n=7;

assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least 28, an intermediate immunostimulatory activity if the IFN-α score is between -5 and 23, and a low immunostimulatory activity if the IFN-α score is at most -5, when n=8;

assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least 30, an intermediate immunostimulatory activity if the IFN-α score is between -5 and 30, and a low immunostimulatory activity if the IFN-α score is at most -9, when n=9;

assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least 1.4909 * n + 22.014, an intermediate immunostimulatory activity if the IFN-α score is between 0.005 * n^2 - 0.2671 * n - 3.5531 and 1.4909 * n + 22.014, and a low immunostimulatory activity if the IFN-α score is at most 0.005 * n^2 - 0.2671 * n - 3.5531, when n is greater than 9,

wherein n is the length of the oligonucleotide.

The present invention also provides a method for assigning the IFN-α score of a RNA oligonucleotide comprising steps (a)-(c) described above.
A single-stranded RNA oligonucleotide of the length $n$ ($n>6$) is broken up into all possible 3mer motifs starting at the 5' end. This will result in a total number of $n-2$ possible 3mer motifs. For example the 20mer ssRNA oligonucleotide 5'-CAGAGCGGGGAUGCGUUGGUC-3' can be broken up into the following 18 3mer motifs (5' -> 3'): CAG, AGA, GAG, AGC, GCG, CGG, GGG, GGA, GAU, AUG, UGC, GCG, CGU, GUU, UUG, UGG, GGU, GUC.

Subsequently, all of the 3mer motifs are checked against the IFN-α point score matrix (Table 7).

Table 7: IFN-α point score matrix

<table>
<thead>
<tr>
<th>3mer motif (5' -&gt; 3')</th>
<th>IFN-α point score</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA</td>
<td>-2</td>
</tr>
<tr>
<td>ACC</td>
<td>-2</td>
</tr>
<tr>
<td>AGA</td>
<td>-2</td>
</tr>
<tr>
<td>AAC</td>
<td>-1</td>
</tr>
<tr>
<td>AUA</td>
<td>-1</td>
</tr>
<tr>
<td>UGG</td>
<td>+1</td>
</tr>
<tr>
<td>GUA</td>
<td>+3</td>
</tr>
<tr>
<td>GUG</td>
<td>+3</td>
</tr>
<tr>
<td>GGU</td>
<td>+4</td>
</tr>
<tr>
<td>UCA</td>
<td>+4</td>
</tr>
<tr>
<td>UUC</td>
<td>+4</td>
</tr>
<tr>
<td>UUU</td>
<td>+5</td>
</tr>
<tr>
<td>AGU</td>
<td>+6</td>
</tr>
<tr>
<td>UUG</td>
<td>+6</td>
</tr>
<tr>
<td>GUC</td>
<td>+8</td>
</tr>
<tr>
<td>UGU</td>
<td>+8</td>
</tr>
<tr>
<td>GUU</td>
<td>+9</td>
</tr>
</tbody>
</table>

Whenever a 3mer motif is present in the IFN-α point score matrix, the listed point score is added to the so-called predicted IFN-α score of the oligonucleotide analyzed. A 3mer motif which is absent from the IFN-α point score matrix has a point score of 0. Thus the
predicted IFN-α score of an oligonucleotide is the sum of IFN-α scores of all 3mer motifs that are present in the IFN-α point score matrix.

For example, for the 20mer ssRNA oligonucleotide 5'-CAGAGCGGGAUGCGUUGGUC-3', a predicted IFN-α score can be calculated as follows:

<table>
<thead>
<tr>
<th>3mer motifs in the 20mer ssRNA</th>
<th>Score in the IFN-α point score matrix</th>
<th>predicted IFN-α score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AGA</td>
<td>(-2)</td>
<td>-2</td>
</tr>
<tr>
<td>GAG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AGC</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GCG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CGG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GGG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GGA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GAU</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AUG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UGC</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GCG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CGU</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GUU</td>
<td>(+9)</td>
<td>+9</td>
</tr>
<tr>
<td>UUG</td>
<td>(+6)</td>
<td>+6</td>
</tr>
<tr>
<td>UGG</td>
<td>(+1)</td>
<td>+1</td>
</tr>
<tr>
<td>GUU</td>
<td>(+4)</td>
<td>+4</td>
</tr>
<tr>
<td>GUC</td>
<td>(+8)</td>
<td>+8</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>+26</td>
</tr>
</tbody>
</table>

This method is herein referred to as the "addition method".

The present application further provides an alternative method for predicting the immunostimulatory activity, in particular the IFN-α-inducing activity, of a RNA oligonucleotide, comprising the steps of:
(a) identifying all possible 3-nucleotide (3mer) motifs contained in the oligonucleotide;
(b) assigning an IFN-α point score for each individual 3mer motif according to Table 12A;
(c) assigning the highest individual IFN-α point score as the IFN-α score of the oligonucleotide; and
(d) assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least 0.58, an intermediate immunostimulatory activity if the IFN-α score is between 0.11 and 0.58, and a low immunostimulatory activity if the IFN-α score is at most 0.11.

The present invention also provides a method for assigning the IFN-α score of a RNA oligonucleotide comprising steps (a)-(c) described above.

This method is herein referred to as the "simplified method".

The IFN-α score of a double-stranded RNA oligonucleotide is the higher of the two IFN-α scores for the two strands.

In the case of a double-stranded or partially double-stranded RNA oligonucleotide, the oligonucleotide is assigned high immunostimulatory activity if at least one of the strands meets the threshold for having high immunostimulatory activity as defined above; the oligonucleotide is assigned low immunostimulatory activity if both strands meet the threshold for having low immunostimulatory activity as defined above. The rest RNA oligonucleotides are assigned intermediate immunostimulatory activity.

**Method for Designing and Preparing RNA Oligonucleotides**

The present application provides a method for preparing a RNA oligonucleotide having immunostimulatory activity, in particular, high IFN-α-inducing activity, comprising the steps of:
(a) providing candidate oligonucleotide sequence(s);
(b) identifying oligonucleotide sequence(s) with high immunostimulatory activity predicted according to the method of prediction described in the previous section;
(c) preparing the RNA oligonucleotide(s) identified for high immunostimulatory activity in step (b); and

(d) optionally testing the immunostimulatory activity of the RNA oligonucleotide(s) prepared in step (c) according to the method of determination described previously; and

(e) further optionally modifying the oligonucleotide(s) to optimize the immunostimulatory activity.

The present application also provides a method for preparing a RNA oligonucleotide having low immunostimulatory activity, in particular, low IFN-α-inducing activity, comprising the steps of:

(a) providing candidate oligonucleotide sequence(s);

(b) identifying oligonucleotide sequence(s) with low immunostimulatory activity predicted according to the method of prediction described in the previous section;

(c) preparing the RNA oligonucleotide(s) identified for low immunostimulatory activity in step (b); and

(d) optionally testing the RNA oligonucleotide(s) prepared in step (c) for the lack of immunostimulatory activity according to the method of determination described previously; and

(e) further optionally modifying the oligonucleotide(s) to minimize the immunostimulatory activity.

The present invention further provides a method for preparing a RNA oligonucleotide having high immunostimulatory activity, in particular, high IFN-α-inducing activity, comprising the steps of:

(a) providing an oligonucleotide sequence which comprises at least one, preferably at least two, more preferably at least three, even more preferably at least four, of the 4-nucleotide (4mer) motifs selected from the group consisting of:

GUUC (No. 1), GUCA (No. 2), GCUC (No. 3), GUUG (No. 4), GUUU (No. 5),
GGUU (No. 6), GUGU (No. 7), GGUC (No. 8), GUCU (No. 9), GUCC (No. 10),
GCUU (No. 11), UUGU (No. 12), UGUC (No. 13), CUGU (No. 14), CGUC (No. 15),
UGUU (No. 16), GUUA (No. 17), UGUA (No. 18), UUUC (No. 19), UGUG (No. 20),
GGUA (No. 21), GUCG (No. 22), UUUG (No. 23), UGGU (No. 24), GUGG (No. 25),
GUGC (No. 26), GUAC (No. 27), GUAU (No. 28), UAGU (No. 29), GUAG (No. 30),
UUCA (No. 31), UUGG (No. 32), UCUC (No. 33), CAGU (No. 34), UUCG (No. 35),
CUUC (No. 36), GAGU (No. 37), GGUG (No. 38), UUGC (No. 39), UUUU (No. 40),
CUCA (No. 41), UCGU (No. 42), UUCU (No. 43), UGGC (No. 44), CGUU (No. 45),
CUUG (No. 46), UUAC (No. 47),
wherein the nucleotide sequences of the motifs are 5' - 3'
wherein the oligonucleotide is between 6 and 64, preferably between 12 and 50,
more preferably between 14 and 40, even more preferably between 16 and 36, and most
preferably between 18 and 25 nucleotides in length,
wherein at least one strand of the RNA oligonucleotide has an IFN-α score of at
least 23 when n=6; at least 26 when n=7; at least 28 when n=8; at least 30 when n=9; at
least 1.4909 x n + 22.014 when n is greater than 9, wherein the IFN-α score is assigned
according to the "addition method" as described above, and wherein n is the length of
the oligonucleotide,
or wherein at least one strand of the RNA oligonucleotide has an IFN-α score of at
least 0.58, wherein the IFN-α score is assigned according to the "simplified method" as
described above,
(b) preparing the RNA oligonucleotide of step (a); and
(c) optionally testing the immunostimulatory activity of the RNA oligonucleotide
prepared in step (b) according to the method of determining the immunostimulatory
activity as described above; and
(d) further optionally modifying the oligonucleotide to optimize the immunostimulatory
activity.

The RNA oligonucleotide can be single-stranded, double-stranded or partially double-
stranded.

The RNA oligonucleotide can have other functionalities such as the gene silencing
activity.

The methods provided by the present application can be used to prepare
immunostimulatory RNA oligonucleotides, siRNA, shRNA or antisense RNA with high or
low immunostimulatory activity.

Some of the RNA oligonucleotides which have low immunostimulatory activity, i.e., the
non-immunostimulatory oligonucleotides, may in fact have inhibitory activity against
immune activation. Such an immunoinhibitory oligonucleotide may be able to prevent
immune activation induced by an immune-stimulatory oligonucleotide when used in combination.

RNA oligonucleotides can be prepared by methods including, but are not limited to, chemical synthesis, in vitro and in vivo transcription from linear templates (e.g., PCR product) and circular templates (e.g., viral or non-viral vectors).

Method for Preparing siRNA Having High or Low Immunostimulatory Activity

The present invention provides a method for preparing an siRNA having gene silencing activity for a target gene and having immunostimulatory activity, in particular, IFN-α-inducing activity, comprising the steps of:

(a) identifying all potential siRNA antisense sequences for a target mRNA;
(b) identifying antisense sequences that have gene silencing activity;
(c) predicting the immunostimulatory activity for the antisense sequences identified in step (b) and their complementary (i.e., sense) sequences;
(d) identifying siRNA wherein at least one of the sense and antisense sequences has an IFN-α score of at least $1.4909 \times n + 31,014$, wherein the IFN-α score is assigned according to the "addition method" described above, wherein $n$ is the length of the sequence and $n$ is between 19 and 25;
(e) decreasing the IFN-α score threshold by 1 if no siRNA is identified in step (d), until at least one siRNA is identified;
(f) preparing the siRNA identified in step (d) or (e);
(g) optionally testing the gene silencing and/or the immunostimulatory activity of the siRNA prepared in (f);
(h) further optionally modify the siRNA prepared in (f) to optimize the gene silencing and/or immunostimulatory activity.

The present invention also provides an alternative method for preparing siRNA with gene silencing activity and immunostimulatory activity, comprising the steps of:

(a) identifying all potential siRNA antisense sequences for a target mRNA;
(b) predicting the immunostimulatory activity for all of the antisense sequences identified in (a) and their complementary (i.e., sense) sequences;
identifying 10 siRNA with the highest IFN-α scores, wherein the IFN-α score is assigned according to the "addition method" described above, wherein the IFN-α score of the siRNA is the higher of the two scores for the sense and the antisense strand;

(d) identifying siRNA with gene silencing activity among the 10 siRNA identified in step (C);

(e) identifying siRNA with the next highest IFN-α scores if no siRNA can be identified in step (d); repeat steps (d) and (e) until at least one siRNA is identified;

(f) preparing the siRNA identified in step (d) or (e);

(g) optionally testing the gene silencing and/or the immunostimulatory activity of the siRNA prepared in (f);

(h) further optionally modify the siRNA prepared in (f) to optimize the gene silencing and/or immunostimulatory activity.

The present invention further provides a method for preparing an siRNA having gene silencing activity for a target gene and having low (or minimal) immunostimulatory activity, in particular, IFN-α-inducing activity, comprising the steps of:

(a) identifying all potential siRNA antisense sequences for a target mRNA;

(b) identifying antisense sequences that have gene silencing activity;

(c) predicting the immunostimulatory activity for the antisense sequences identified in step (b) and their complementary (i.e., sense) sequences;

(d) identifying siRNA wherein both the sense and the antisense sequences have an IFN-α score of at most 0.6075 × n - 9.9484, wherein the IFN-α score is assigned according to the "addition method" described above, wherein n is the length of the sequence and n is between 19 and 25;

(e) increasing the IFN-α score threshold by 1 if no siRNA is identified in step (b), until at least one siRNA is identified;

(f) preparing the siRNA identified in step (d) or (e);

(g) optionally testing the gene silencing and/or the immunostimulatory activity of the siRNA prepared in (f);

(h) further optionally modify the siRNA prepared in (f) to optimize the gene silencing activity and/or to minimize the immunostimulatory activity.
The present invention also provide an alternative method for preparing siRNA with gene silencing activity and low (or minimal) immunostimulatory activity, comprising the steps of:

(a) identifying all potential siRNA antisense sequences for a target mRNA;
(b) predicting the immunostimulatory activity for all of the antisense sequences identified in (a) and their complementary (i.e., sense) sequences;
(c) identifying 10 siRNA with the lowest IFN-α scores, wherein the IFN-α score is assigned according to the "addition method" described above, wherein the IFN-α score of the siRNA is the higher of the two scores for the sense and the antisense strand;
(d) identifying siRNA with gene silencing activity among the 10 siRNA identified in step (c);
(e) identifying 10 siRNA with the next lowest IFN-α scores if no siRNA can be identified in step (d); repeat steps (d) and (e) until at least one siRNA is identified;
(f) preparing the siRNA identified in step (d) or (e);
(g) optionally testing the gene silencing and/or the immunostimulatory activity of the siRNA prepared in (f);
(h) further optionally modify the siRNA prepared in (f) to optimize the gene silencing activity and/or to minimize the immunostimulatory activity.

Candidate siRNA with gene silencing activity for a given gene can be identified using methods known to those skilled in the art, including, but not limited to, commercial engines such as those available from Dharmacon, Qiagen, Invitrogen. Furthermore, the gene silencing activity of an siRNA may be predicted using the algorithm of Reynolds et al. (2004, Nat Biotechnol 22:326-330) and may be determined experimentally.

The gene silencing activity of an siRNA can be determined experimentally by methods well known in the art. For Example, the RNA oligonucleotide may be introduced into a cell by a method known in the art such as transfection and transduction; the mRNA level of the target gene can be determined by routine methods such as Northern blot analysis, quantitative PCR, RNase protection assay, and branching DNA; and the protein expression level can be determined by routine methods such as Western blotting, ELISA, and biological activity assays specific to the target protein. Furthermore, the mRNA level of all known and hypothetical genes can be determined at the global level using the
microarray technology. Technologies in the field of proteonomics allow for the protein levels of a large number of genes to be determined at the global level as well.

The same methods may be used for preparing an shRNA which comprises the sense and the antisense sequences of an siRNA identified by the above methods and a loop sequence. The preferred loop sequences for shRNA are known to those skilled in the art.

The same methods may also be used for preparing antisense RNA wherein only one strand, the antisense strand, needs to be identified and prepared.

The siRNA, shRNA and antisense RNA can be prepared by methods including, but are not limited to, chemical synthesis, in vitro and in vivo transcription from PCR products and viral or non-viral vectors.

**Immunostimulatory RNA Oligonucleotides**

The present invention provides an immunostimulatory RNA oligonucleotide having immunostimulatory activity, in particular, IFN-α-inducing activity, comprising at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, and most preferably at least six, of the motifs selected from the group consisting of GUY, GUNY, and GNUY, wherein Y is a pyrimidine (either a U or a C), wherein N is any one of nucleotides A, C, G, and U, and wherein the nucleotide sequences of the motifs are 5' -> 3'.

In one embodiment, the immunostimulatory RNA oligonucleotide is between 6 and 64, preferably between 12 and 50, more preferably between 14 and 40, even more preferably between 16 and 36, and most preferably between 18 and 25 nucleotides in length.

In a further embodiment, at least one strand of the immunostimulatory RNA oligonucleotide has an IFN-α score of at least 23 when n=6; at least 26 when n=7; at least 28 when n=8; at least 30 when n=9; at least 1.4909 x n + 22.014 when n is greater than 9, wherein the IFN-α score is assigned according to the "addition method"
described above, wherein \( n \) is the length of the oligonucleotide, provided that the oligonucleotide of the present invention is not 5'-UUGAUGUUGUUGUGUCUA-3' (Judge et al. 2005, Nat Biotechnol 23:457-462), 5'-GCACCACUAGUUGGUUGUC-3' (Sioud 2005, J Mol Biol 348:1079-1090), 5'-GUUGUAGUUGUACUCCAGC-3' (Sioud), 5'-GCCCGUCUGUUGUGUGACUC-3' (Heil et al. 2004 Science 303:1526-1529), 5'-GUCUGUUGUGUG-3' (Heil, et al.), 5'-GUUGUGGGUUGGUGUGUGUG-3' (WO 03/086280).

In an alternative embodiment, at least one strand of the immunostimulatory RNA oligonucleotide has an IFN-\( \alpha \) score of at least 0.58, wherein the IFN-\( \alpha \) score is assigned according to the "simplified method" described above, provided that the oligonucleotide of the present invention is not 5'-UUGAUGUUGUUGUGUCUA-3' (Judge et al. 2005, Nat Biotechnol 23:457-462), 5'-GCACCACUAGUUGGUUGUC-3' (Sioud 2005, J Mol Biol 348:1079-1090), 5'-GUUGUAGUUGUACUCCAGC-3' (Sioud), 5'-GCCCGUCUGUUGUGUGACUC-3' (Heil et al. 2004 Science 303:1526-1529), 5'-GUCUGUUGUGUG-3' (Heil, et al.), 5'-GUUGUGGGUUGGUGUGUGUG-3' (WO 03/086280).

The present invention further provides an immunostimulatory RNA oligonucleotide having immunostimulatory activity, in particular, IFN-\( \alpha \)-inducing activity, comprising at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, and most preferably at least six, of the 4-nucleotide (4mer) motifs selected from the group consisting of:

GUUC (No. 1), GUCA (No. 2), GCUC (No. 3), GUUG (No. 4), GUUU (No. 5),
GGUU (No. 6), GUGU (No. 7), GGUC (No. 8), GUCU (No. 9), GCC (No. 10),
GCUU (No. 11), UUGU (No. 12), UGUC (No. 13), CUGU (No. 14), CGUC (No. 15),
UGUU (No. 16), GUUA (No. 17), UGUA (No. 18), UUUC (No. 19), UGUG (No. 20),
GGUA (No. 21), GUCG (No. 22), UUGU (No. 23), UGGU (No. 24), GUGG (No. 25),
GUGC (No. 26), GUAC (No. 27), GUAA (No. 28), UAGU (No. 29), GUAG (No. 30),
UUCA (No. 31), UUGG (No. 32), UCUC (No. 33), CAGU (No. 34), UUCG (No. 35),
CUUC (No. 36), GAGU (No. 37), GGUG (No. 38), UUGC (No. 39), UUUA (No. 40),
CUCA (No. 41), UCGU (No. 42), UUCU (No. 43), UGGC (No. 44), CGUU (No. 45),
CUUG (No. 46), UUAC (No. 47),
wherein the nucleotide sequences of the motifs are 5' -> 3'.
wherein the oligonucleotide is between 6 and 64, preferably between 12 and 50, more preferably between 14 and 40, even more preferably between 16 and 36, and most preferably between 18 and 25 nucleotides in length,

wherein at least one strand of the RNA oligonucleotide has an IFN-α score of at least 23 when \( n=6 \); at least 26 when \( n=7 \); at least 28 when \( n=8 \); at least 30 when \( n=9 \); at least 1.4909 \( \times n + 22.014 \) when \( n \) is greater than 9, wherein the IFN-α score is assigned according to the "addition method" described above,

or wherein at least one strand of the RNA oligonucleotide has an IFN-α score of at least 0.58, wherein the IFN-α score is assigned according to the "simplified method" described above,

wherein \( n \) is the length of the oligonucleotide,

and wherein the oligonucleotide is not 5′-UUGAUGUGUUAGUGCUA-3′ (Judge et al. 2005, Nat Biotechnol 23:457-462), 5′-GCACCACUAGUGGUUGUC-3′ (Sioud 2005, J Mol Biol 348:1079-1090), 5′-GUUGUAGUUGACUC-3′ (Sioud), 5′-GCCGUCUGUGUGUGACUC-3′ (Heil et al. 2004 Science 303:1526-1529), 5′-GUCUGUUGUG-3′ (Heil, et al.), 5′-GUUGUGGUUGUGUGUGUGUG-3′ (WO 03/086280).

In one embodiment, the 4mer motifs are selected from the group consisting of No. 1-19, No. 1-18, No. 1-17, No. 1-16, preferably, No. 1-15, No. 1-14, No. 1-13, No. 1-12, more preferably, No. 1-11, No. 1-10, No. 1-9, No. 1-8, No. 1-7, even more preferably, No. 1-6, No. 1-5, No. 1-4, No. 1-3, most preferably, No. 1-2 of the 4mer motifs.

The immunostimulatory RNA oligonucleotide of the invention may comprise one or more copies of the same 4mer motif, or one or more copies of different 4mer motifs.

The present invention also provide an immunostimulatory RNA oligonucleotide having immunostimulatory, in particular, IFN-α-inducing activity, comprising at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, most preferably at least six, of the 4mer motifs selected from the group consisting of No. 1-6, preferably No. 1-5, No. 1-4, No. 1-3, more preferably No. 1-2 of the 4mer motifs, wherein the spacer nucleotides which are not part of any of the 4mer motif(s) are identical, and wherein the spacer nucleotide is selected from the group consisting of A, T, C, G and variants and derivatives thereof.
In one embodiment, the spacer nucleotide is A or a derivative thereof.

In one embodiment, the immunostimulatory RNA oligonucleotide of the invention can comprise one or more copies of one type of 4mer motif (e.g., GUUC) on a poly A backbone. Examples of such an oligonucleotide includes, but are not limited to:

AAAAAAAAGUUCAAAAA
AAAGUUCAAAAAAGUUC
AAAAAAGUCAAAAAAGUUC
GUUCAAGUCAAGUCA

In another embodiment, the immunostimulatory RNA oligonucleotide of the invention can comprise one or more copies of more than one type of 4mer motif (e.g., GUUC GUCA, GCUC, GUUG, GUUU, GGUU) on a poly A backbone. Examples of such an oligonucleotide includes, but are not limited to:

AGUUCAAAAGUCAAGCUC
AGUUCAGUUCAAGUCAAGCUC
AAAGUUCAAAAGUCAAAAGCUCAAGUUGAAAGUUUAAAGGUUAAA

The more than one 4mer motifs in an immunostimulatory RNA oligonucleotide may overlap. For example, AAAAGU UGCUCAAAAAA.

Examples of immunostimulatory RNA oligonucleotides of the invention include, but are not limited to:

aaaguucaaaaaaguucaaa (SEQ ID NO:391)
aaagucaaaaaaaguucaaa (SEQ ID NO:392)
aaagcucaaaaaaguucaaa (SEQ ID NO:393)
aaagguugaaaaaguucaaa (SEQ ID NO:394)
aaaguuuaaaaaguucaaa (SEQ ID NO:395)
aaagguuuaaaaaaguucaaa (SEQ ID NO:396)
aaaguguuaaaaaaguucaaa (SEQ ID NO:397)
aaagguuuaaaaaaguucaaa (SEQ ID NO:398)
aaagucuaaaaaaaguucaaa (SEQ ID NO:399)
aaaguccaaaaaaaguucaaa (SEQ ID NO:400)
aaaguuuuaaaaaaguucaaa (SEQ ID NO:401)
aaagucaaaaaaagucaaaa (SEQ ID NO:402)
aaagcucaaaaaaagucaaaa (SEQ ID NO:403)
aaaguugaaaaaagucaaaa (SEQ ID NO:404)
aaaguuuuaaaaaagucaaaa (SEQ ID NO:405)
aaagguuuaaaaaagucaaaaa (SEQ ID NO:406)
aaaguguuaaaaaaagucaaaa (SEQ ID NO:407)
aaagucuuiuaaaaaaagucaaaa (SEQ ID NO:408)
aaagucuuuaaaaaaagucaaaa (SEQ ID NO:409)
aaaguccaaaaaaaagucaaaa (SEQ ID NO:410)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:411)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:412)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:413)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:414)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:415)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:416)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:417)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:418)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:419)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:420)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:421)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:422)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:423)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:424)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:425)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:426)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:427)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:428)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:429)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:430)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:431)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:432)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:433)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:434)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:435)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:436)
aaagucuuaaaaaaagucaaaa (SEQ ID NO:437)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:438)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:439)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:440)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:441)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:442)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:443)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:444)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:445)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:446)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:447)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:448)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:449)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:450)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:451)
aaagucuaaaaaaaaaguuuaa (SEQ ID NO:452)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:453)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:454)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:455)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:456)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:457)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:458)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:459)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:460)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:461)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:462)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:463)
aaagucuaaaaaaaaaguuuaa (SEQ ID NO:464)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:465)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:466)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:467)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:468)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:469)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:470)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:471)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:472)
aaagucuaaaaaaaaaguuuaaa (SEQ ID NO:473)
In one embodiment, immunostimulatory RNA oligonucleotide of the invention does not have gene silencing activity for any known mammalian gene.

The immunostimulatory RNA oligonucleotide of the invention may be single-stranded, single-stranded containing a self-complementary sequence and can form a hairpin structure, double-stranded, or partially double-stranded.

Furthermore, the immunostimulatory RNA oligonucleotide of the invention may be covalently linked to one or more lipophilic groups which enhance the stability and the activity and facilitate the delivery of the RNA oligonucleotides.

As used herein, the term "lipophilic" or "lipophilic group" broadly refers to any compound or chemical moiety having an affinity for lipids. Lipophilic groups encompass compounds of many different types, including those having aromatic, aliphatic or alicyclic characteristics, and combinations thereof.
In specific embodiments, the lipophilic group is an aliphatic, alicyclic, or polyalicyclic substance, such as a steroid (e.g., sterol) or a branched aliphatic hydrocarbon. The lipophilic group generally comprises a hydrocarbon chain, which may be cyclic or acyclic. The hydrocarbon chain may comprise various substituents and/or at least one heteroatom, such as an oxygen atom. Such lipophilic aliphatic moieties include, without limitation, saturated or unsaturated fatty acids, waxes (e.g., monohydric alcohol esters of fatty acids and fatty diamides), terpenes (e.g., the diterpenes, C_{15} sesquiterpenes, C_{20} diterpenes, C_{30} triterpenes, and C_{40} tetraterpenes), and other polyalicyclic hydrocarbons.

The lipophilic group may be attached by any method known in the art, including via a functional grouping present in or introduced into the RNA oligonucleotide, such as a hydroxy group (e.g., -CO—CH_{2} -OH). Conjugation of the RNA oligonucleotide and the lipophilic group may occur, for example, through formation of an ether or a carboxylic or carbamoyl ester linkage between the hydroxy and an alkyl group R—, an alkanoyl group RCO— or a substituted carbamoyl group KNHCO—. The alkyl group R may be cyclic (e.g., cyclohexyl) or acyclic (e.g., straight-chained or branched; and saturated or unsaturated). Alkyl group R may be a butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl or octadecyl group, or the like. Preferably, the lipophilic group is conjugated to the 5'-hydroxyl group of the terminal nucleotide. In a preferred embodiment, the lipophilic group is 12-hydroxydodecanoic acid bisdecylamide.

In another embodiment, the lipophilic group is a steroid, such as sterol. Steroids are polycyclic compounds containing a perhydro-1,2-cyclopentanophenanthe nine ring system. Steroids include, without limitation, bile acids (e.g., cholic acid, deoxycholic acid and dehydrocholic acid), cortisone, digoxigenin, testosterone, cholesterol and cationic steroids, such as cortisone.

In a preferred embodiment, the lipophilic group is cholesterol or a derivative thereof. A "cholesterol derivative" refers to a compound derived from cholesterol, for example by substitution, addition or removal of substituents. The steroid may be attached to the RNA oligonucleotide by any method known in the art. In a preferred embodiment, the lipophilic group is cholesteryl (6-hydroxyhexyl) carbamate.

In another embodiment, the lipophilic group is an aromatic moiety. In this context, the term "aromatic" refers broadly to mono- and polyaromatic hydrocarbons. Aromatic
groups include, without limitation, C$_6$-C$_{14}$ aryl moieties comprising one to three aromatic rings, which may be optionally substituted; "aralkyl" or "arylalkyl" groups comprising an aryl group covalently linked to an alkyl group, either of which may independently be optionally substituted or unsubstituted; and "heteroaryl" groups. As used herein, the term "heteroaryl" refers to groups having 5 to 14 ring atoms, preferably 5, 6, 9, or 10 ring atoms; having 6, 10, or 14π electrons shared in a cyclic array; and having, in addition to carbon atoms, between one and about three heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and sulfur (S).

As used herein, a "substituted" alkyl, cycloalkyl, aryl, heteroaryl, or heterocyclic group is one having between one and about four, preferably between one and about three, more preferably one or two, non-hydrogen substituents. Suitable substituents include, without limitation, halo, hydroxy, nitro, haloalkyl, alkyl, alkaryl, aralkyl, alkoxy, aryloxy, amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalky, alkoxycarbonyl, carboxy, hydroxycarbonyl, alanethiol, alanesulfonyl, arenesulfonamide, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, and ureido groups.

The lipophilic group can be covalently linked directly or indirectly via a linker to the RNA oligonucleotide. The covalent linkage may or may not comprise a phosphodiester group. And the linker may be of various lengths. The preferred lengths of the linker are known to those skilled in the art and may be determined experimentally.

In one embodiment, the lipophilic group is covalently linked to the 5' end of at least one strand of the RNA oligonucleotide.

In addition, the immunostimulatory oligonucleotide of the invention may be coupled to a solid support. By "coupled" it is meant that the oligonucleotide is covalently or non-covalently, directly or indirectly, linked to the solid support. Suitable solid supports include, but are not limited to, silicon wafers, synthetic polymer support such as polystyrene, polypropylene, polyglycidylmethacrylate, substituted polystyrene (e.g., aminated or carboxylated polystyrene, polyacrylamides, polyamides, polyvinylchlorides, etc.), glass, agarose, nitrocellulose, nylon and gelatin nanoparticles. Solid support may enhance the stability and the activity of the oligonucleotide, especially short oligonucleotides less than 16 nucleotides in length.
Immunostimulatory RNA Oligonucleotide Conjugates

The present invention also provides immunomodulatory RNA oligonucleotide conjugates, comprising an immunomodulatory RNA oligonucleotide and an antigen conjugated to the oligonucleotide. In some embodiments, the antigen is conjugated to the oligonucleotide at a position other than its 3' end. In some embodiments, the antigen produces a vaccine effect.

The antigen is preferably selected from the group consisting of disease/disorder-related antigens. The disorder may be a cancer, a dermatological disorder, an immune disorder, a metabolic disorder, a neurological disorder, an ocular disease, an infection, or other hereditary and non-hereditary disorders. The antigen may be a protein, a polypeptide, a peptide, a carbohydrate, or a combination thereof.

The immunostimulatory RNA oligonucleotide may be covalently linked to the antigen, or it is otherwise operatively associated with the antigen. As used herein, the term "operatively associated with" refers to any association that maintains the activity of both the oligonucleotide and the antigen. Non-limiting examples of such operative associations include being part of the same liposome or other such delivery vehicle or reagent. In embodiments wherein the oligonucleotide agent is covalently linked to the antigen, such covalent linkage preferably is at any position on the oligonucleotide that does not interfere with the immunostimulatory activity of the oligonucleotide.

RNA Oligonucleotide with Gene Silencing Activity

The present invention provides a RNA oligonucleotide with gene silencing activity.

In one embodiment, the RNA oligonucleotide has both gene silencing activity and immunostimulatory activity, wherein at least one strand of the oligonucleotide has an IFN-α score of at least 1.4909 x n + 31.014, wherein the IFN-α score is assigned according to the "addition method" described above, wherein n is the length of the oligonucleotide.
In an alternative embodiment, the RNA oligonucleotide has both gene silencing activity and immunostimulatory activity, wherein at least one strand of the oligonucleotide has an IFN-α score of at least 0.58, wherein the IFN-α score is assigned according to the "simplified method" described above.

In another embodiment, the RNA oligonucleotide has gene silencing activity and low/minimal immunostimulatory activity, wherein all strand(s) of the oligonucleotide has(have) an IFN-α score of at most 0.6075 x n - 9.9484, wherein the IFN-α score is assigned according to the "addition method" described above, wherein n is the length of the oligonucleotide.

In an alternative embodiment, the RNA oligonucleotide has gene silencing activity and low/minimal immunostimulatory activity, wherein all strand(s) of the oligonucleotide has(have) an IFN-α score of at most 0.11, wherein the IFN-α score is assigned according to the "simplified method" described above.

The RNA oligonucleotide may be an siRNA, an shRNA or an antisense RNA. The siRNA is between 14 and 25 nucleotides in length; the shRNA is between 30 and 70 nucleotides in length; and the antisense RNA is between 14 and 50 nucleotides in length.

In the case of an immunostimulatory siRNA, the gene silencing activity resides on the antisense strands which is complementary to the target mRNA, whereas the immunostimulatory activity may reside on either the sense or the antisense strand. In a preferred embodiment, the immunostimulatory activity resides on the sense strand; i.e., the sense strand has an IFN-α score of at least 1.4909 x n + 31.014 when the IFN-α score is assigned according to the "addition method" described above or the sense strand has and IFN-α score of at least 0.58 when the IFN-α score is assigned according to the "simplified method" described above.

In the case of an shRNA, the molecule is processed inside a cell to yield a siRNA molecule and the loop (linker) sequence. Therefore, the IFN-α score needs to be calculated not only for the intact molecule (a single-stranded RNA), but also for both strands of the resulting siRNA molecule and the single-stranded loop sequence. The shRNA is considered to have high immunostimulatory activity if at least one of the above-mentioned entities has an IFN-α score above the threshold of 1.4909 x n + 31.014.
according to the "addition method" or 0.58 according to the "simplified method"; the molecule is considered to have low immunostimulatory activity if all of the entities mentioned above have an IFN-α score below the threshold of 0.6075 x n - 9.9484 according to the "addition method" or 0.11 according to the "simplified method". The gene silencing activity resides in the sequence that corresponds to the antisense strand of the siRNA; whereas the immunostimulatory activity may reside in any part of the molecule. In a preferred embodiment, the immunostimulatory activity resides in the portion of an shRNA that corresponds to the sense strand of the corresponding siRNA or in the loop sequence; i.e., said portion has an IFN-α score of at least 1.4909 x n + 31.014 according to the "addition method" or 0.58 according to the "simplified method".

In the case of an immunostimulatory antisense RNA, the immunostimulatory activity and the gene silencing activity have to reside on the same strand.

The gene silencing RNA oligonucleotide of the invention may be covalently linked to one or more lipophilic groups which enhance the stability and the activity and facilitate the delivery of the RNA oligonucleotides.

**Pharmaceutical Compositions**

The present invention provides pharmaceutical compositions comprising one or more of the RNA oligonucleotides of the invention and a pharmaceutically acceptable carrier. The more than one RNA oligonucleotides may have the same, similar, or different functionalities including, but are not limited to immunostimulatory activity and gene silencing activity.

For example, a RNA oligonucleotide having immunostimulatory activity but lacking gene silencing activity may be combined with a RNA oligonucleotide having gene silencing activity and low immunostimulatory activity in a pharmaceutical composition to achieve both immune activation and gene silencing. Such a combination composition may be useful for treating disorders such as cancers and viral infections. Such a combination composition may be necessary when the two activities cannot be optimized on a single RNA oligonucleotide.
In one embodiment, the pharmaceutical composition further comprises a RNA complexation agent. In a preferred embodiment, the complexation agent is a polycationic peptide, preferably poly-L-arginine (poly-L-Arg). In a preferred embodiment, the polycationic peptide, in particular, poly-L-Arg, is at least 24 amino acids in length. The polycationic peptide, in particular, poly-L Arg, may be a heterogeneous mixture of peptides of different lengths.

The pharmaceutical composition of the invention may further comprises another agent such as an agent that stabilizes the RNA oligonucleotide(s), e.g., a protein that complexes with the oligonucleotide agent to form an iRNP. Still other agents include chelators, e.g., EDTA (e.g., to remove divalent cations such as Mg^{2+}), salts, RNase inhibitors (e.g., a broad specificity RNase inhibitor such as RNAsin) and so forth.

The pharmaceutical composition of the present invention can further comprise one or more additional pharmaceutically active (or therapeutic) agents which are selected from the group consisting of agents that are used for the treatment of cancer, dermatological disorders, immune disorders, metabolic disorders, neurological disorders, ocular diseases, infections, and other hereditary and non-hereditary disorders in a mammal.

In certain embodiments, the additional pharmaceutically active agent is selected from the group consisting of immunostimulatory RNA oligonucleotides, immunostimulatory DNA oligonucleotides, cytokines, chemokines, growth factors, antibiotics, anti-angiogenic factors, chemotherapeutic agents, anti-viral agents, anti-fungal agents, anti-parasitic agents, and antibodies. In one embodiment, the additional pharmaceutically active agent is natural or recombinant IFN-α polypeptide, or a CpG-containing or non-CpG-containing DNA oligonucleotide capable of inducing IFN-α (see e.g., WO 01/22990, WO 03/101375). In another embodiment, the additional pharmaceutically active agent is natural or recombinant IL-12, or an immunostimulatory RNA oligonucleotide capable inducing IL-12 (see e.g. our co-pending application; Sugiyama et al. 2005, J Immunol 174:2273-2279). In yet another embodiment, the additional pharmaceutically active agent is an anti-angiogenic factor such as vasostatin or an anti-VEGF antibody. In certain embodiments, the additional pharmaceutically active agent is a cancer-specific agent such as Herceptin®, Rituxan®, Gleevec®, Iressa®.
A formulated oligonucleotide composition can assume a variety of states. In some examples, the composition is at least partially crystalline, uniformly crystalline, and/or anhydrous (e.g., less than 80, 50, 30, 20, or 10% water). In another example, the oligonucleotide agent is in an aqueous phase, e.g., in a solution that includes water, this form being the preferred form for administration via inhalation.

The aqueous phase or the crystalline compositions can be incorporated into a delivery vehicle, e.g., a liposome (particularly for the aqueous phase), or a particle (e.g., a microparticle as can be appropriate for a crystalline composition). Generally, the oligonucleotide composition is formulated in a manner that is compatible with the intended method of administration.

The pharmaceutical compositions encompassed by the invention may be administered by any means known in the art including, but not limited to, oral or parenteral routes, including intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, airway (aerosol), ocular, rectal, vaginal, and topical (including buccal and sublingual) administration. In preferred embodiments, the pharmaceutical compositions are administered by intravenous or intraparentral infusion or injection. The pharmaceutical compositions can also be administered intraparenchymally, intrathecal\(^\d\), and/or by stereotactic injection.

For oral administration, the oligonucleotide agent useful in the invention will generally be provided in the form of tablets or capsules, as a powder or granules, or as an aqueous solution or suspension.

Tablets for oral use may include the active ingredients mixed with pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavoring agents, coloring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatin, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material such as glyceryl monostearate or glyceryl distearate, to delay absorption in the gastrointestinal tract.
Capsules for oral use include hard gelatin capsules in which the active ingredient is mixed with a solid diluent, and soft gelatin capsules wherein the active ingredient is mixed with water or an oil such as peanut oil, liquid paraffin or olive oil.

For intramuscular, intraperitoneal, subcutaneous and intravenous use, the pharmaceutical compositions of the invention will generally be provided in sterile aqueous solutions or suspensions, buffered to an appropriate pH and isotonicity. Suitable aqueous vehicles include Ringer's solution and isotonic sodium chloride. In a preferred embodiment, the carrier consists exclusively of an aqueous buffer. In this context, "exclusively" means no auxiliary agents or encapsulating substances are present which might affect or mediate uptake of oligonucleotide agent in the cells that harbor the target gene or virus. Such substances include, for example, micellar structures, such as liposomes or capsids, as described below. Although microinjection, lipofection, viruses, viroids, capsids, capsoids, or other auxiliary agents are required to introduce oligonucleotide agent into cell cultures, surprisingly these methods and agents are not necessary for uptake of oligonucleotide agent in vivo. The oligonucleotide agent of the present invention are particularly advantageous in that they do not require the use of an auxiliary agent to mediate uptake of the oligonucleotide agent into the cell, many of which agents are toxic or associated with deleterious side effects. Aqueous suspensions according to the invention may include suspending agents such as cellulose derivatives, sodium alginate, polyvinylpyrrolidone and gum tragacanth, and a wetting agent such as lecithin. Suitable preservatives for aqueous suspensions include ethyl and n-propyl p-hydroxybenzoate.

The pharmaceutical compositions can also include encapsulated formulations to protect the oligonucleotide agent against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811; PCT publication WO 91/06309; and European patent publication EP-A-43075.
In general, a suitable dose of a RNA oligonucleotide will be in the range of 0.001 to 500 milligrams per kilogram body weight of the recipient per day (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 100 milligrams per kilogram, about 1 milligrams per kilogram to about 75 milligrams per kilogram, about 10 micrograms per kilogram to about 50 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram). The pharmaceutical composition may be administered once per day, or the oligonucleotide agent may be administered as two, three, four, five, six or more sub-doses at appropriate intervals throughout the day. In that case, the oligonucleotide agent contained in each sub-dose must be correspondingly smaller in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, e.g., using a conventional sustained release formulation which provides sustained release of the oligonucleotide agent over a several day period. Sustained release formulations are well known in the art. In this embodiment, the dosage unit contains a corresponding multiple of the daily dose.

The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the infection or disease/disorder, previous treatments, the general health and/or age of the subject, and other diseases/disorders present. Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment or a series of treatments. Estimates of effective dosages and in vivo half-lives for the individual RNA oligonucleotide agent encompassed by the invention can be made using conventional methodologies or on the basis of in vivo testing using an appropriate animal model, as described elsewhere herein.

Toxicity and therapeutic efficacy of the RNA oligonucleotide and the pharmaceutical composition of the invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. oligonucleotide agents that exhibit high therapeutic indices are preferred.
The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosages of compositions of the invention are preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any oligonucleotide agent used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range of the oligonucleotide agent or, when appropriate, of the polypeptide product of a target sequence (e.g., achieving a decreased concentration of the polypeptide) that includes the IC50 (i.e., the concentration of the test oligonucleotide agent which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

The administering physician can adjust the amount and timing of the administration of the pharmaceutical composition of the invention on the basis of results observed using standard measures of efficacy known in the art or described herein.

**Use of the RNA Oligonucleotide for Inducing an Immune Response**

The present application provides the use of the immunostimulatory RNA oligonucleotide of the invention for the preparation of a pharmaceutical composition for inducing an immune response in a mammal.

Inducing an immune response means initiating or causing an increase in one or more of B-cell activation, T-cell activation, natural killer cell activation, activation of antigen presenting cells (e.g., B cells, dendritic cells, monocytes and macrophages), cytokine production, chemokine production, specific cell surface marker expression, in particular, expression of co-stimulatory molecules. In one aspect, such an immune response involves the production of type I IFN, in particular, IFN-α, in cells such as PDC.
Use of the RNA Oligonucleotide for Treating Diseases/Disorders

The present invention provides the use of the immunostimulatory RNA oligonucleotide of the invention for the preparation of a pharmaceutical composition for preventing and/or treating a disorder selected from immune disorders, infections, and cancers in a mammal, wherein the induction of an immune response is beneficial to the mammal.

The present invention also provides the use of the RNA oligonucleotide of the invention which has both immunostimulatory activity and gene silencing activity for the preparation of a pharmaceutical composition for preventing and/or treating a disorder selected from infections and cancers in a mammal, wherein the induction of an immune response together with the downregulation of a disorder-related gene are beneficial to the mammal.

The present invention further provides the use of the RNA oligonucleotide of the invention which has gene silencing activity and low/minimal immunostimulatory activity for the preparation of a pharmaceutical composition for preventing and/or treating a disorder in a mammal caused by the expression or overexpression of a disorder-related gene, wherein the induction of an immune disorder it to be avoided. The disorder may be selected from cancer, dermatological disorders, immune disorders, metabolic disorders, neurological disorders, ocular diseases, infections, and other hereditary and non-hereditary disorders.

The immune disorders include, but are not limited to, allergy, autoimmune disorders, inflammatory disorders, and immunodeficiency.

Allergies include, but are not limited to, food allergies and respiratory allergies.

Autoimmune diseases include, but are not limited to, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, encephalomyelitis, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjogren's Syndrome, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal
reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing, loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves' disease, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

Inflammatory disorders include, without limitation, airway inflammation which includes, without limitation, asthma.

Immunodeficiencies include, but are not limited to, spontaneous immunodeficiency, acquired immunodeficiency (including AIDS), drug-induced immunodeficiency (such as that induced by immunosuppressants used in transplantation and chemotherapeutic agents used for treating cancer).

In one embodiment, the immune disorders include those caused by pathological Th2 responses.

The infections include, but are not limited to viral infections, bacterial infections, anthrax, parasitic infections, fungal infections and prion infection.

Viral infections include, but are not limited to, infection by hepatitis C, hepatitis B, herpes simplex virus (HSV), HIV-AIDS, poliovirus, and smallpox virus. Examples of (+) strand RNA viruses which can be targeted for inhibition include, without limitation, picomaviruses, caliciviruses, nodaviruses, coronaviruses, arteriviruses, flaviviruses, and togaviruses. Examples of picomaviruses include enterovirus (poliovirus 1), rhinovirus (human rhinovirus 1A), hepatovirus (hepatitis A virus), cardiovirus (encephalomyocarditis virus), aphthovirus (foot-and-mouth disease virus O), and parechovirus (human echovirus 22). Examples of caliciviruses include vesiculovirus (swine vesicular exanthema virus), lagovirus (rabbit hemorrhagic disease virus), "Norwalk-like viruses" (Norwalk virus), "Sapporo-like viruses" (Sapporo virus), and "hepatitis E-like viruses" (hepatitis E virus). Betanodavirus (striped jack nervous necrosis virus) is the representative nodavirus. Coronaviruses include coronavirus (avian infections bronchitis virus) and torovirus (Berne virus). Arterivirus (equine arteritis virus) is the representative arteriviridus. Togavirises include alphavirus (Sindbis virus) and rubivirus (Rubella virus).
Finally, the flaviviruses include flavivirus (Yellow fever virus), pestivirus (bovine diarrhea virus), and hepacivirus (hepatitis C virus).

In certain embodiments, the viral infections are selected from chornic hepatitis B, chornic hepatitis C, HIV infection, RSV infection, HSV infection, VSV infection, CMV infection, and influenza infection.

Cancers include, but are not limited to biliary tract cancer, brain cancer, breast cancer, cervical cancer, choriocarcinoma, colon cancer, endometrial cancer, esophageal cancer, gastric cancer, intraepithelial neoplasm, leukemia, lymphoma, liver cancer, lung cancer, melanoma, myelomas, neuroblastoma, oral cancer, ovarian cancer, pancreatic cancer, prostate cancer, rectal cancer, sarcoma, skin cancer, testicular cancer, thyroid cancer and renal cancer.


Dermatological disorders include, but are not limited to, psoriasis, acne, rosacea, eczema, molluscum contagious, seborrheic keratosis, actinic keratosis, verruca vulgaris.

Metabolic disorders include, but are not limited to, diabetes and obesity.

Ocular diseases include, but are not limited to, age-related macular degeneration.

Neurological disorders include, but are not limited to, Alzheimer's disease, Huntington's disease, Parkinson's disease, and spinal cord injury.

Hereditary diseases include, but are not limited to, cystic fibrosis.
In one embodiment, the pharmaceutical composition is for administration selected from the group consisting of airway, oral, ocular, parenteral (including intravenous, intradermal, intramuscular, intraperitoneal, and subcutaneous), rectal, vaginal and topical (including buccal and sublingual) administration.

In another embodiment, the pharmaceutical composition is for use in combination with one or more treatments of disorders selected from treatments for cancer, dermatological disorders, immune disorders, metabolic disorders, neurological disorders, ocular diseases, infections, and other hereditary and non-hereditary disorders in a mammal. Such treatments include, but are not limited to, surgery, chemotherapy, radiation therapy, and the administration of pharmaceutically active (or therapeutic) agents such as immunostimulatory RNA oligonucleotides, immunostimulatory DNA oligonucleotides, cytokines, chemokines, growth factors, antibiotics, anti-angiogenic factors, chemotherapeutic agents, anti-viral agents, anti-fungal agents, anti-parasitic agents, and antibodies.

In one embodiment, pharmaceutically active agent is natural or recombinant IFN-α polypeptide, or a CpG-containing or non-CpG-containing DNA oligonucleotide capable of inducing IFN-α (see e.g., WO 01/22990, WO 03/101375). In another embodiment, the pharmaceutically active agent is natural or recombinant IL-12, or an immunostimulatory RNA oligonucleotide capable inducing IL-12 (see e.g. our co-pending application; Sugiyama et al. 2005, *J Immunol* 174:2273-2279). In yet another embodiment, the pharmaceutically active agent is an anti-angiogenic factor such as vasostatin or an anti-VEGF antibody. In certain embodiments, the pharmaceutically active agent is a cancer-specific agent such as Herceptin, Rituxan, Gleevec, Iressa.

Mammals include, but are not limited to, rats, mice, cats, dogs, horses, sheep, cattle, cows, pigs, rabbits, non-human primates, and humans. In a preferred embodiment, the mammal is human.

**In Vitro Method for Inducing IFN-α Production**

The present invention provides an in vitro method of inducing IFN-α production in a mammalian cell, comprising the steps of:
(a) complexing an immunostimulatory RNA oligonucleotide of the invention with a complexation agent; and
(b) contacting the cell with the complex prepared in step (a).

The mammalian cell is capable of producing IFN-α. In one embodiment, the mammalian cell expresses TLR7, TLR8, or both TLR7 and TLR8. The mammalian cells include, but are not limited to, peripheral blood mononuclear cells (PBMC), plasmacytoid dendritic cells (PDC), myeloid dendritic cells (MDC), B cells, macrophages, monocytes, natural killer cells, granulocytes, endothelial cells, cell lines such as THP1, and cells containing exogenous DNA which directs the expression of TLR7 or TLR8 or both TLR7 or TLR8 such as transfected CHO, HEK293 or COS cells.

In one embodiment of the invention, the complexation agent is a polycationic peptide, preferably poly-L-arginine (poly-L-Arg). In one embodiment, the polycationic peptide, in particular, poly-L-Arg, is at least 24 amino acids in length. The polycationic peptide, in particular, poly-L-Arg, may be a heterogeneous mixture of peptides of different lengths.

In a preferred embodiment, the mammal is human.

**In Vitro Method for Activating Mammalian Dendritic Cells**

The present application provides an in vitro method of activating mammalian dendritic cells, comprising the steps of:

(a) complexing an immunostimulatory RNA oligonucleotide of the invention with a complexation agent;
(b) contacting dendritic cells isolated from a donor mammal with the complexed RNA oligonucleotide; and
(c) contacting the dendritic cells with an antigen.

In one embodiment of the invention, the complexation agent is a polycationic peptide, preferably poly-L-arginine (poly-L-Arg). In one embodiment, the polycationic peptide, in particular, poly-L-Arg, is at least 24 amino acids in length. The polycationic peptide, in particular, poly-L-Arg, may be a heterogeneous mixture of peptides of different lengths.
In one embodiment, the antigen is a disease/disorder-related antigen. The disorder may be a cancer, a dermatological disorder, an immune disorder, a metabolic disorder, a neurological disorder, an ocular disease, an infection, or other hereditary and non-hereditary disorders. The antigen may be a protein, a polypeptide, a peptide, a carbohydrate, or a combination thereof.

The present invention further provides the use of the in vitro activated dendritic cells for the preparation of a medicament for inducing an immune response in a mammal, wherein the in vitro activated dendritic cells are for transfer into a recipient that is the same or different from the donor.

In a preferred embodiment, the mammal is human.

The present invention is illustrated by the following examples.

**Examples**

**Example 1.** Stimulation of PBMC using poly-L-arginine-complexed single-stranded RNA oligonucleotides

First we sought to develop an assay system for the comparison of the IFN-α inducing capacity of single-stranded RNA (ssRNA) oligonucleotides on a large scale. Peripheral blood mononuclear cells (PBMC) were selected as a biological system that is relevant for future clinical application. Within PBMC, the plasmacytoid dendritic cell (PDC) is responsible for IFN-α production upon stimulation with ssRNA oligonucleotides. In order to stimulate IFN-α production in PDC, ssRNA oligonucleotides require transfection. While cationic lipids such as lipofectamine are well-established to support IFN-α production in isolated PDC, the induction of IFN-α in PBMC was not satisfactory. In order to improve transfection of RNA in PBMC we tested a number of cationic polypeptides including poly-L-Lys, poly-His and poly-L-Arg. Among these three, poly-L-Arg (14 kD) was the most potent polycatonic peptide and was even more potent than the cationic lipid lipofectamine to support the stimulatory activity of an established immunostimulatory RNA oligonucleotide (RNA9.2sense) (Fig. 1). Within PBMC, the source of IFN-α
production were found to be PDC, since PDC depletion abrogated IFN-α production (data not shown). Importantly, poly-L-Arg complexation maintained the same sequence dependency (sense versus antisense strand) of ssRNA oligonucleotide-mediated IFN-α induction that was previously seen for lipofectamine (Hornung et al. 2005, *Nat Med* 11:263-270). This experimental system was found to be robust, since adding different concentrations of RNA oligonucleotide from 80 % to 120 % had little effect on the amount of IFN-α induced. This is in marked contrast to the use of cationic lipids that require exact adjustment of the net charges to avoid cytotoxicity. Together these data indicated that poly-L-Arg complexation is an effective and reliable system to screen for ssRNA oligonucleotide-induced IFN-α production in PBMC.

The experimental procedures are described in more detail in the following:

**PBMC isolation**

Human PBMC were prepared from whole blood donated by young healthy donors. PBMC were obtained from whole blood by Ficoll-Hypaque density gradient centrifugation (Biochrom, Berlin, Germany). PBMC were cultured in RPMI-Medium (Biochrom, Berlin, Germany) supplemented with human AB-Serum (2 vol%, Firma, Germany) at a density of 2x10^6 PBMC/ ml. Subsequently PBMC were plated into 96-well flat bottom wells at 200 µl/ well. Cells were kept on ice until stimulation.

**Resuspension and annealing of ssRNA oligonucleotides**

Lyophilized ssRNA oligonucleotides (Eurogentec, Belgium) were resuspended in sterile, RNase free water at a concentration of 620 µg/ml. Subsequently 40 µl of the ssRNA oligonucleotides, 40 µl sterile, RNase free water and 20 µl 5x annealing buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA) were combined. This resulted in a final volume of 100 µl with a final concentration of the ssRNA oligonucleotide of 248 µg/ml. Subsequently the solution was incubated at 95°C for 2 minutes and cooled down to 20°C with a linear decrease in temperature in 60 minutes (-1.25°C/1 minute). The solution was then stored at -80°C prior to usage (ssRNA oligonucleotide stock solution).

**Stimulation**

If not otherwise indicated, ssRNA oligonucleotides were complexed using the polycationic polymer Poly-L-arginine Hydrochloride with a molecular weight of 5.000 -15.000 (Prod. Number: P4663, Sigma, Munich, Germany). Lyophilized Poly-L-arginine
Hydrochloride was dissolved in sterile, RNase free water at a concentration of 2000 µg/ml (Poly-L-arginine stock solution) and subsequently aliquoted and stored at a temperature of -20°C. ssRNA oligonucleotides were complexed using the following protocol:

1. The poly-L-arginine stock solution and the ssRNA oligonucleotide stock solution are thawed to 4°C.
2. Poly-L-arginine is diluted in Phosphate buffered saline to a concentration of 24 µg/ml (1:83.3 dilution from 2000 µg/ml stock solution).
3. Immediately the ssRNA oligonucleotide is added to a final concentration of 14.8 µg/ml (1:16.6 dilution from 248 µg/ml stock solution).
4. Subsequently, the solution is mixed thoroughly by vortexing for 10 seconds.
5. Next, 15µl of this solution is added immediately to 200µl PBMC in the 96-well flat bottom plate. The final concentration of poly-L-arginine is 1.67 µg/ml, whereas the final concentration of the ssRNA oligonucleotide is 1.03 µg/ml.

Throughout all procedures the temperature was kept between 20 - 25°C. On all 96-well plates PBMC from three individual donors were plated, whereas eleven different ssRNA oligonucleotides were tested. In addition, on each plate the ssRNA oligonucleotide 9.2 sense (5’-AGCUUAACCUGUCCUCAA-3’) was included as a positive control. All tested ssRNA oligonucleotides were run in duplicates. After stimulation cells were cultured at 37°C / 5% CO2 for 44 hours.

**ELISA**

After 44 hours of cell culture supematants were collected and stored in three individual aliquots at -80 0C. Prior to ELISA-procedures frozen supematants were thawed at 20 - 25°C for two hours. To measure IFN-a the IFN-a module set Bender MedSystems (Prod. Number: BMS216MST, Graz, Austria) was used. This ELISA detects most of IFN-α isoforms at a detection range of 8-500 pg/ml. All ELISA procedures were performed according to manufacturer’s recommendations.

**Example 2.** Rational design of a 4mer-motif library to screen for potent motifs within ssRNA
Previous experiments have shown that a minimal length of 19 bases is required for maximal IFN-α induction by ssRNA oligonucleotides. Since poly adenosine oligonucleotides proved to be inactive in terms of IFN-α induction, we decided to generate a ssRNA oligonucleotide library by placing putative motifs into the center of poly adenosine RNA oligonucleotides. In a first set of experiments we determined the minimal length of a motif for IFN-α induction in our system. We designed a panel of 19mer poly-adenosine ssRNA oligonucleotides with increasing numbers of uracil in the center: 5’-U-3’, 5’-UU-3’, 5’-UUU-3’ and 5’-UUUU-3”. These oligonucleotides were compared to the previously published standard ssRNA oligonucleotide RNA9.2sense (5’-AGCUUAACCUGUCCUCAA-S’): while the 1mer motif 5’-U-3’ hardly induced IFN-α (0.8 % of RNA9.2sense), considerable amounts of IFN-α were observed for the 2mer motif 5’-UU-3’ (10.6 % of RNA9.2sense), the 3mer motif 5’-UUU-3’ (15.7 % of RNA9.2sense) and the 4mer motif 5’-UUUU-3’ (50.5 % of RNA9.2sense) (Fig. 2). Since the largest increment of IFN-α induction was seen between the 3mer and 4mer motif, we generated a library of ssRNA oligonucleotides comprising all possible 4mer motifs in the centre of a poly adenosine oligonucleotide. In view of the fact that the flanking adenosine residues can be part of a 4mer RNA sequence motif, only 193 ssRNA oligonucleotides (table 1) were needed to cover all 256 possible 4mer motifs.

Example 3. Generation and processing of raw data

All 193 ssRNA oligonucleotides were tested on PBMC of six individual healthy donors using poly-L-Arg for complexation. At 44 hours after stimulation with RNA oligonucleotides, supernatants were collected and IFN-α production was measured by ELISA. Prior to statistical analysis the raw data were processed as follows: for each cell culture plate the mean IFN-α value of the experimental duplicates for each tested ssRNA oligonucleotide were normalized to the ssRNA oligonucleotide RNA9.2sense (5’-AGCUUAACCUGUCCUCAA-S’). This standard RNA oligonucleotide was included as a positive control on all cell culture plates. Normalization was performed by calculating the ratio of IFN-α induced by the tested oligonucleotide and IFN-α induced by the standard oligonucleotide RNA9.2sense. Thus, for each tested oligonucleotide in an individual donor a mean ratio of IFN-α induction was obtained. In the following, this mean of the ratios is referred to as IFN-a index (one value of IFN-a index per donor). For example testing ssRNA oligonucleotide ANP144 (5’-AAAAAAGUUGA-3’-3) in donor 1
gave the mean of the two raw values of the duplicates (IFN-α in supernatant) of 2024 pg/ml, whereas the control oligonucleotide RNA9.2sense (5’-AGCUUAACCUGUCCUCAA-3’) resulted in 1256 pg/ml. The corresponding IFN-α index of oligonucleotide ANP144 for donor 1 was calculated to be 1.61 (= 2024 pg/ml divided by 1256 pg/ml).

Next, the means of all IFN-α indices for every individual donor were calculated. Then the adjusted IFN-α indices were calculated as IFN-α index minus the mean of all IFN-α indices of one individual donor. For example ssRNA oligonucleotide ANP144 of donor 1 (5’-AAAAAAAGUUGAAAAAAAA-3’) had an IFN-α index of 1.61, whereas the mean of all IFN-a indices of donor 1 was 0.37. The adjusted IFN-a index of ANP144 was calculated: 1.61 minus 0.37 = 1.24 (= subtracting 0.37 from). The adjusted IFN-α indices from all six donors were summarized by calculating the means and the corresponding standard error of mean. The data are depicted in ascending order (Fig. 3A, B, C, D). The adjusted IFN-a indices of the top thirty ssRNA oligonucleotides of all six donors were compared using a two-tailed Student’s t-test (Fig. 3E). For most combinations tested, a significant difference was observed when the interval between the analyzed pairs was at least six or seven places in the assortment.

The ssRNA oligonucleotides were split into two groups: group 1 (table 2) comprising all ssRNA oligonucleotides with an IFN-α index that lower than the mean IFN-α index off all ssRNA oligonucleotides (or in other words with a mean of the adjusted IFN-α index below 0) (table 1), and group 2 (table 3) comprising all ssRNA oligonucleotides with an IFN-a index higher than the mean IFN-α index off all tested ssRNA oligonucleotides (or in other words with a mean of the adjusted IFN-α index below 0) (table 1). Thus group 1 contained all ssRNA oligonucleotides with an adjusted IFN-α index below 0, whereas group 2 contained all ssRNA oligonucleotides with an adjusted IFN-a index above 0. Group 1 consisted of 148 ssRNA oligonucleotides (75 %), whereas group 2 consisted of 45 ssRNA oligonucleotides (25 %).

**Example 4.** Analyzing the predictive value of 1mer, 2mer and 3mer motifs within the 4mer motif matrix
Next the frequency of 1mer motifs (5'-X-3'), 2mer motifs (5'-XX-3', 5'-X*X-3', 5'-X**X-3') and 3mer motifs (5''-XXX-3', 5'-XX*X-3', 5'-X'XX-3') in ssRNA oligonucleotides with an IFN-α index below the mean IFN-α index (group 1) and above the mean IFN-α index (group 2) was analyzed. Multiple occurrences of a motif within one single ssRNA oligonucleotide was accounted for. For example, the motif 5'-GU-3' was present twice in the ssRNA oligonucleotide ANP142 (5'-AAAAAAGUGUAAAAAAAA-S', group 2). Consequently the ssRNA oligonucleotide ANP142 contributed two counts for motif 5'-GU-3' within group 2. In order to compare the distribution of a specific motif between the two groups, the relative occurrence of a specific motif was calculated (ratio of the absolute number of occurrence of a specific motif over the total number of occurrence of all motifs). For example the motif 5'-GU-3' was found 7 times in group 1 (total number of 5'-XX-3'-motifs in group 1: 2482) and 33 times in group 2 (total number of 5'-XX-3'-motifs in group 2: 799). Thus the calculated relative occurrence for motif 5'-GU-3' in group 1 was 0.0028, whereas the respective relative occurrence for group 2 was 0.0413. In figure 4 the relative occurrence of 1mer motifs (5'-X-3'), 2mer motifs (5'-XX-3', 5'-X*X-3', 5'-X**X-3') and 3mer motifs (5'-XXX-3', 5'-XX*X-3', 5'-X'XX-3') within the two groups is shown. A significant overrepresentation or underrepresentation of a given motif was analyzed using a chi-square test. The null hypothesis of equal distribution within both groups was rejected when the calculated p-value was below 0.05 (significant differences in distribution are indicated by "***" in Fig. 4).

**Example 5.** Calculating an individual IFN-α score for 1mer-, 2mer- and 3mer-motifs

A mean IFN-α index for all possible 1mer motifs (5'-X-3'), 2mer motifs (5'-XX-3', 5'-X*X-3', 5'-X**X-3') or 3mer motifs (5'-XXX-3', 5'-XX*X-3', 5'-X'XX-3') was obtained by calculating a mean IFN-α index of all ssRNA oligonucleotides containing the corresponding motifs. This mean IFN-α index is referred to as the IFN-α score of a given motif. For example the 3mer motif 5'-GUC-3' was contained in ssRNA oligonucleotides ANP 35, 83, 131, 137, 138, 139 and 179 with respective adjusted IFN-α indices of 1.33, 0.68, 0.93, 0.79, 0.44, 0.84, 0.73. The IFN-α score of the 3mer motif 5'-GUC-3' was thus calculated to be 0.82 with a standard error of mean of 0.11. The calculation of the IFN-α score of a motif did not account for the position of the motif within the sequence of the corresponding ssRNA oligonucleotides. Multiple occurrences of one motif within the same ssRNA oligonucleotide was accounted for by adding the corresponding IFN-α
index times the number of its occurrence within the oligonucleotide to the calculation of the corresponding IFN-α score of the motif. Consequently an IFN-α score could be assigned to all possible 1mer motifs (5'-X-3'), 2mer motifs (5'-XX-3', 5'-X*X-3\ 5'-X**X-3') or 3mer motifs (5'-XXX-3\ 5'-XX*X-3\ 5'-X*XX-3') (Fig. 5).

**Example 6.** Predicting the IFN-α index of ssRNA oligonucleotides using the IFN-α score of 1mer-, 2mer- and 3mer-motifs

Next we tested the predictive value of the calculated 1mer-, 2mer- and 3mer-motif IFN-α scores to predict ssRNA oligonucleotides with a low or high IFN-α index. Thus for each ssRNA oligonucleotide the occurrence of a set of motifs was tested and the respective IFN-α scores were assigned to the ssRNA. For example for the panel of 3mer motifs with unspaced sequences (5'-XXX-3') the ssRNA oligonucleotide ANP 35 (5'-AAAAAAAAGUCAAAAAAAA-3') was analyzed the following way:

<table>
<thead>
<tr>
<th>3mer motif (5'-XXX-3')</th>
<th>IFN-α score</th>
<th>Occurrences within the ssRNA oligonucleotide ANP 35</th>
<th>Assigned IFN-α score</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-AAA-3'</td>
<td>-0.0041</td>
<td>12</td>
<td>-0.0503</td>
</tr>
<tr>
<td>5'-AAG-3'</td>
<td>+0.0990</td>
<td>1</td>
<td>+0.0990</td>
</tr>
<tr>
<td>5'-AGU-3'</td>
<td>+0.5796</td>
<td>1</td>
<td>+0.5796</td>
</tr>
<tr>
<td>5'-GUC-3'</td>
<td>+0.8115</td>
<td>1</td>
<td>+0.8115</td>
</tr>
<tr>
<td>5'-UCA-3'</td>
<td>+0.3668</td>
<td>1</td>
<td>+0.3668</td>
</tr>
<tr>
<td>5'-CAA-3'</td>
<td>+0.0033</td>
<td>1</td>
<td>+0.0033</td>
</tr>
<tr>
<td>overall</td>
<td></td>
<td></td>
<td>+1.8099</td>
</tr>
</tbody>
</table>

All ssRNA oligonucleotides were assigned an individual IFN-α score for all possible motif-combinations (1mer-, 2mer- and 3mer-motifs). Next, the prediction that was obtained by using the assigned IFN-α scores was compared to the actual adjusted IFN-α indices for all ssRNA oligonucleotides and for each motif combination. Data were sorted in ascending order according to the adjusted IFN-α indices. For all predictions, the correlation coefficient was calculated: Using the IFN-α scores of 1mer motifs (5'-X-3') to predict the actual adjusted IFN-α indices off all ssRNA oligonucleotides a correlation coefficient of 0.53 was obtained (Figure 6A). When 2mer motifs were used to predict the
adjusted IFN-α indices a correlation coefficient of 0.77 was obtained for 5'-XX-3'-motifs, a correlation coefficient of 0.58 for 5'-X*X-3'-motifs and a correlation coefficient of 0.60 for 5'-X**X-3'-motifs (Figure 6B). Using 3mer motifs to predict the adjusted IFN-α indices, a correlation coefficient of 0.87 was calculated for 5'-XXX-3'-motifs, of 0.80 for 5'-XX*X-3'-motifs and of 0.80 for 5'-X*X*X-3'-motifs.

Next, the most accurate prediction algorithm (using the individual IFN-α scores of the 5'-XXX-3'-motifs) was translated into a point score system. The matrix of the 64 different 3mer motifs (5'-XXX-3') was reduced to encompass only the motifs that were significantly over- or underrepresented in either group 1 or group 2 ssRNA oligonucleotides. Even though only 17 3mer motifs (5'-XXX-3') were left in this matrix, a correlation coefficient of 0.87 could still be calculated when using the respective IFN-α scores to predict the IFN-α indices of the complete ssRNA oligonucleotide library (data not shown). In addition, the respective IFN-α scores of the reduced 3mer motif matrix was translated into a point score system by assigning a point score to each 3mer motif (Figure 6D). Using this point score system, a prediction of the measured IFN-α indices of all 193 ssRNA oligonucleotides was calculated with a corresponding correlation coefficient of 0.87 (Figure 6E).

**Example 7.** Influence of the positioning and the flanking bases on the IFN-α-inducing activity of a potent 4mer motif within a 19mer ssRNA oligonucleotide

Next we sought to address the impact of moving a potent 4mer motif to either the 5' or the 3' end within a 19mer ssRNA oligonucleotide. A panel of ssRNA oligonucleotides was designed that included the potent 4mer motif 5'-GUCA-3' within a 19mer poly-A ssRNA oligonucleotide (Table 4). The positioning of the 4mer motif was chosen to be either 2, 6, 8, 10 or 14 bases from the 5'-end of the ssRNA oligonucleotide. PBMC were stimulated with the respective ssRNA oligonucleotides and IFN-α production was assessed 44 hours after stimulation. Positioning the 4mer motif two bases from the 5'-end of the ssRNA oligonucleotide slightly decreased IFN-α induction by 13%, when compared to the center positioning (8 bases from the 5'-end). A higher decrease in IFN-α production (29%) was seen, when the 4mer motif was positioned on the 3'-end of the ssRNA oligonucleotide (14 bases from the 5'-end). Nevertheless all ssRNA
oligonucleotides tested were still more potent in terms of IFN-α induction than the positive control 9.2sense (Figure 7A).

In addition, we addressed the influence of flanking bases on the stimulatory activity of the potent 4mer motif 5'-GUCA-3'. A panel of 16 ssRNA oligonucleotides, which included all possible oligonucleotides with permuted bases at the flanking positions to the 5' and the 3'-end of the central 5'-GUCA-3'-motif (table 5), was tested (Figure 7B). As soon as the 5'-GUCA-3'-motif was modified with a preceding 5'-C-3', a significant decrease to less than 50% of the original activity was obtained (Figure 7C). In addition, when a 5'-U-3' preceded the 5'-GUCA-3'-motif, a significant decrease to about 77% of the original activity was observed (Figure 7C). In contrast, changing the preceding base to a 5'-G-3' did not impact on the stimulatory activity of the 5'-GUCA-3'-motif. The modification of the flanking base to the 3'-end had little impact on the stimulatory activity of the 5'-GUCA-3'-motif. Nevertheless, a slight, yet significant decrease in activity was detected, as soon as the base to the 3'-end was changed into a 5'-C-3'.

Example 8. Predicting the immunostimulatory activity of complex 19mer ssRNA oligonucleotides using the 3mer motif 5'-XXX-3' based IFN-α point score matrix.

Next, we employed the IFN-α point score matrix to predict the potency of complex (i.e., no longer on a poly A backbone) 19mer ssRNA oligonucleotides in terms of IFN-α induction. Modified versions of RNA9.2sense that have been previously described (Hornung et al. 2005, Nat Med 11:263-270) were used to stimulate PBMC; IFN-α production was measured 44 h after stimulation. In addition, respective sequences were analyzed using the above-described algorithm that is based on the IFN-α score of 3mer motifs 5'-XXX-3'. Both the measured and the predicted data were normalized to the ssRNA oligonucleotide RNA9.2sense (set to 100%). In Fig. 8A both measured data and predicted data are depicted. The correlation coefficient for this analysis was calculated to be 0.84.

Example 9. Validation of the method of prediction

Heil and colleagues (Heil F et al. 2004, *Science* 303: 1526-1529) found that when added to human PBMC as a complex with cationic lipid DOTAP, RNA40 (5'-GCC CGU CUG UUG UGU GAC UC-3', HIV-1 U5 region nt 108-127) but not RNA41 (U-A replacement of RNA40) or RNA42 (G-A replacement of RNA40) induced IFN-\(\alpha\), and that the source of IFN-\(\alpha\) was PDC. In contrast, both RNA40 and RNA42, but not RNA41 induced TNF-\(\alpha\), IL-6 and IL-12p40; TNF-\(\alpha\) was produced by CD11c+ cells. Similar results were found with isolated murine PDC and macrophages. RNA33 (5-GUGUGUGUG-3') and RNA34 (5'-GUCUGUUGUGUG-3'), both containing one phosphorothioate linkage at the 3'end, induced the same cytokine profile as RNA40 in the mouse system. Heil et al. stated that a sequence motif responsible for the IFN-\(\alpha\) inducing activity of the RNA oligonucleotides tested could not be identified; subsequently, the activity was attributed to the high GU content of the sequence. Our analysis of the results of Heil et al. reveals that RNA40 contains a 4mer motif, GUUG, which is the fourth most potent motif in inducing IFN-\(\alpha\) production in our matrix. Furthermore, our 3mer-based algorithm predicts high IFN-\(\alpha\)-inducing activity for RNA40, RNA33 and RNA34, but not for RNA41 and RNA42, which is in agreement with the experimental data (Figure 8D).

Another publication on ssRNA and IFN-\(\alpha\) in the human system is from our own group (Hornung V et al. 2005, *Nat Med* 11: 263-270). In this publication, we identified a 9mer sequence motif which was responsible for the immunostimulatory activity of the ssRNA oligonucleotide RNA9.2 sense (5'-AGC UUA ACC UGU CCU UCA A-3', 9mer motif underlined). No motif shorter than the 9mer motif was characterized. The analysis of our previously published results reveals that the previously identified 9mer motif contains GUCC which is the tenth most potent immunostimulatory 4mer in our matrix. Furthermore, our 3mer-based IFN-\(\alpha\) point score matrix predicts RNA9.2 sense to be a highly active IFN-\(\alpha\)-inducing sequence. Moreover, our 3mer-based IFN-\(\alpha\) point score matrix offers a prediction of the IFN-\(\alpha\)-inducing activities of other sequences tested in Hornung et al.; our prediction correlates very well with the published experimental data.
Another study in the human system was published by Sioud and colleagues (Sioud M et al. 2005, J Mol Biol 348: 1079-1090). The authors examined a panel of 32 siRNAs for their ability to induce TNF-α and IL-6 in PBMC. The most active sequence was number 27 (sense: 5'-GUCCGGGCAGGUCUACUUUTT-3') either as siRNA (double-stranded) or as the sense strand. As negative control, number 32 (sense: 5'-GCUGGAGAUCCUGAAGAACTT-3') was used. Of note, the whole panel was not screened for IFN-α-inducing activity; only sequence 27 was assayed for IFN-α induction in PBMC. Both the number 27 siRNA and the corresponding sense strand were found to induce IFN-α. DOTAP was used for transfection. Our analysis of the panel of 32 siRNAs reveals that only oligonucleotide number 27, but none of the other oligonucleotides of the panel, contains the motif GUCC, which is ranked number 10 on our most potent immunostimulatory 4mer list and which is also contained in the 9mer motif of our earlier paper (Homung V et al. 2005, Nat Med 11: 263-270) discussed above. Furthermore, our 3mer-based IFN-α point score matrix predicts potent IFN-α-inducing activity for siRNA number 27. However, since siRNA number 27 was the only sequence examined for IFN-α-inducing activity in Sioud et al, a comprehensive analysis of the whole panel of siRNAs could not be carried out.

Besides our own previous publication (Homung V et al. 2005, Nat Med 11: 263-270), Judge and colleagues are the only ones who proposed a sequence motif (UGUGU) for the IFN-α-inducing activity of RNA oligonucleotides (Judge AD et al. 2005, Nat Biotechnol 2005. 23: 457-462). Although most of their work was done with siRNA (double-stranded), for one of their potent immunostimulatory sequences, β-Gal control, both the sense and the antisense strand were tested. The sense strand, but not the antisense strand, was found to be active to inducing IFN-α in human PBMC. The sense strand (5'-UUGAUGUUUAGUCGCUA-3') contained the proposed UGUGU motif, while the antisense strand (5'-UAGCGACUAACACACUAACAAA-3') did not. The introduction of one (UGCGU) or two (UGCGC) mismatches in the sense strand sequence of the β-Gal control siRNA led to the loss of IFN-α-inducing activity. On the other hand, the creation of the UGUGU motif, starting from UGGCU, in a primarily non-stimulatory siRNA, BP1, led to an enhanced IFN-α-inducing activity. Furthermore, Judge and colleagues showed that they could select non-stimulatory siRNA sequences by avoiding U-rich sequences and GUGU motifs. Indeed, in our 4mer matrix, GUGU is the 7th most active motif, and UGUG is the 20th most active motif. Furthermore, the relative IFN-α-
inducing activities of β-Gal control SiRNA, BP1 siRNA and their derivatives predicated by
our 3mer-based IFN-α point score matrix correlates extremely well with the experimental
data of Judge et al. (Figure 8B).

Additional publication reports the induction of IFN-α by RNA oligonucleotides in the
mouse system.

Barchet W et al. (2005, Eur J Immunol 35: 236-242) reports IFN-α induction by RNA
oligonucleotides in murine PDC. In this study, the RNA sequences examined were
derived from the 5' and 3' untranslated regions (UTR) of Influenza virus. The following
sequences were used:

5' UTR: 5'-AGUAGAAACAAGGUAGUUU-3' (19mer)
3' UTR: 5'-UUACCUACCUCUUUGCU-3' (19mer)
5'3" UTR: 5'-AGUAGAAACAAGGUAGUUUUUGUUACCUACCUCUUUCGU-3' (42mer),
5' UTR U-C replacement: 5'-AGCAGAAACAAGGCAGCCC-3' (19mer)
5' UTR G-C replacement: 5'-ACUACAAACAACCUU C UUUUUGCU-3' (19mer)

5'UTR, 3'UTR and 5'3'UTR oligonucleotides all induced IFN-α production from murine
PDC. The activity of 5'UTR was significantly reduced when the Gs were replaced by Cs,
and abolished when the Us were replaced by Cs. No motif responsible for the IFN-α-
inducing activity was defined in this study. According to our 4mer motif matrix, 5' UTR
contains the 5th active motif GUUU, and 3' UTR contains the motif UUUU which is above
average. The activity levels of the oligonucleotides used in Barchet et al. predicted by our
3mer-based IFN-α point score matrix correlates with the experimental data of Barchet et
al.

It should be noted that in some studies double stranded RNA oligonucleotides were
used. In such cases, a mean value for the individually analyzed single strands was
calculated. Nevertheless, using the prediction algorithm, a good estimate of the actual
IFN-α data could be obtained.

All of the publications discussed above validate the use of our 4mer matrix and
algorithms for predicting the immunostimulatory activity of RNA oligonucleotides. The
teaching in the prior art with regard to the prediction of IFN-α-inducing activity of RNA
oligonucleotides has been limited. The only criteria available so far are the content of G
and U (Heil et al. 2004, Science 303: 1526-1529), and the presence of the GUGU motif (Judge et al. 2005, Nat Biotechnol 2005. 23: 457-462). With our 3mer-based IFN-α point score matrix, we now can predict the immunostimulatory activity of any RNA oligonucleotide reliably.

**Example 10.** Determining the threshold for high and low immunostimulatory activity

The immunostimulatory activity of any given RNA oligonucleotide can be predicted using the 3mer-based IFN-α point score matrix as described previously (i.e., the "addition method"). For research and drug discovery and development purposes, two groups of RNA oligonucleotide are of interest: Group A oligonucleotides which have high or maximal IFN-α-inducing activity, and Group B oligonucleotides which have low or minimal IFN-α-inducing activity. Among all possible ssRNA oligonucleotides of a certain length, 1% of the oligonucleotides with the highest IFN-α scores are assigned to Group A; where as 1% of the oligonucleotides with the lowest IFN-α scores are assigned to Group B. The cut-off IFN-α score for Group A oligonucleotide is the threshold for high or maximal immunostimulatory activity; the cut-off IFN-α score for Group B oligonucleotide is the threshold for low or minimal immunostimulatory activity.

The IFN-α score thresholds for high/maximal and low/minimal immunostimulatory activity for 19mer ssRNA oligonucleotides are determined as follows:

A pool of all possible sequences of 19mer RNA oligonucleotides consists of $4^{19} = 274,877,906,944$ oligonucleotides. The IFN-α score for every single RNA oligonucleotide in the pool is calculated using the 3mer-based IFN-α point score matrix. All $4^{19}$ oligonucleotides are ranked based on their calculated predicted IFN-α scores. The threshold for group A is determined to be

$$1.4909 \times n + 22.014$$

($n =$ length of the ssRNA oligonucleotide, and $n >9$).

All ssRNA oligonucleotides with a calculated IFN-α score above the threshold value are grouped into Group A. The Group A threshold for 19mer ssRNA oligonucleotides is 50.341 1. Non-limiting examples of Group A 19mer ssRNA oligonucleotides include the following:
The threshold for Group B oligonucleotides is determined to be:

\[(0.005 \times n^2) - (0.2671 \times n) - 3.5531\]

\[(n = \text{length of the ssRNA oligonucleotide, and } X > 9)\]

All ssRNA oligonucleotides with a calculated IFN-α score below the threshold value are grouped into Group B. The Group B threshold for 19mer ssRNA oligonucleotide is -6.823. Non-limiting examples of Group B 19mer oligonucleotide include the following:

<table>
<thead>
<tr>
<th>Sequence (5' --&gt;3')</th>
<th>Predicted IFN-α score</th>
</tr>
</thead>
<tbody>
<tr>
<td>GUGUGUUGCUUGAUUGCC</td>
<td>60</td>
</tr>
<tr>
<td>UUGUAGUUCGUUGCUAGUG</td>
<td>60</td>
</tr>
<tr>
<td>AGUUCAGGGUGGGUUGUAC</td>
<td>62</td>
</tr>
<tr>
<td>UGUUAAGUUGUUCUACCC</td>
<td>62</td>
</tr>
<tr>
<td>AAGUUUGAUAUUUUCAGUA</td>
<td>63</td>
</tr>
<tr>
<td>AGGCGUUGUGUUGCGGGUU</td>
<td>65</td>
</tr>
<tr>
<td>AGAUGUGUAGGGUGUUUU</td>
<td>66</td>
</tr>
<tr>
<td>UAGUGUGUGUCAGUGUGAC</td>
<td>71</td>
</tr>
<tr>
<td>GGUUGCGUGUGGAUGUGUU</td>
<td>72</td>
</tr>
<tr>
<td>UGUAGUUUGUUGAGAGUCA</td>
<td>75</td>
</tr>
<tr>
<td>GUGUGUUGUGCUUGUGUCA</td>
<td>77</td>
</tr>
</tbody>
</table>

For ssRNA oligonucleotides between 3 and 9 nucleotides in length, the Group A and Group B threshold values are given below in Table 8:
Table 8. Threshold IFN-α scores for Group A and Group B oligonucleotides 3-9 nucleotides in length.

<table>
<thead>
<tr>
<th>ssRNA oligonucleotide length</th>
<th>The predicted IFN-α score (using the IFN-α point score matrix)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Threshold for GROUP B ssRNA oligonucleotides</td>
</tr>
<tr>
<td>3</td>
<td>-2</td>
</tr>
<tr>
<td>4</td>
<td>-2</td>
</tr>
<tr>
<td>5</td>
<td>-3</td>
</tr>
<tr>
<td>6</td>
<td>-4</td>
</tr>
<tr>
<td>7</td>
<td>-4</td>
</tr>
<tr>
<td>8</td>
<td>-5</td>
</tr>
<tr>
<td>9</td>
<td>-5</td>
</tr>
</tbody>
</table>

**Example 11.** Designing siRNA with high or low immunostimulatory activity

The threshold for Group A siRNA is

\[ 1.4909 \times n + 31.014 \]

\((n = \text{length of the ssRNA oligonucleotide} \text{ and } n > 9)\) according to the "addition method".

An siRNA is considered a Group A siRNA, i.e., an siRNA with high or maximal immunostimulatory activity, if at least one of the strands, preferably the sense strand, has an IFN-α score above the threshold.

In order to maximize the chance of identifying at least one siRNA with optimized gene silencing as well as immunostimulatory activity, at least ten siRNA molecules need to be identified whose sense strands have an IFN-α score above the threshold for Group A siRNA. When fewer than ten siRNA can be identified that fit the above criteria, the threshold for Group A siRNA is decrease by 1 in a stepwise manner until ten siRNA can be identified.
Most commonly used siRNA are at least 19 nucleotides in length. The threshold for Group A siRNA for a 19mer is 59.341.

The following example demonstrates the identification of Group A siRNA for mRNA of human cyclophilin B (hCyPB) (Accession No. M6087).

For hCyPB, 833 putative or potential siRNA duplexes (19mer) can be identified. The IFN-α score is calculated for both the sense and the antisense strand of all possible 833 19mer siRNA using the IFN-α point score matrix. All siRNA duplexes which contain at least one strand with an IFN-α score higher than 59.341 are put into Group A. 11 siRNA duplexes are assigned to Group A because the IFN-α scores of their sense strands are above the threshold; 24 siRNA duplexes are assigned to Group A because the IFN-α scores of their antisense strands are above the threshold. The Group A hCyPB siRNA are listed in Table 9:

<table>
<thead>
<tr>
<th>Sense strand (5'-&gt; 3')</th>
<th>IFN-α score</th>
<th>Antisense strand (5'-&gt; 3')</th>
<th>IFN-α score</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAACAAACUCCUACCAACA</td>
<td>-9</td>
<td>UGUUGGUAGGAGUUUGUUA</td>
<td>74</td>
</tr>
<tr>
<td>AACAAACUCCUACCAACAC</td>
<td>-9</td>
<td>GUGUUGGUAGGAGUUUGUU</td>
<td>77</td>
</tr>
<tr>
<td>UACCAACACUGACCAAUAA</td>
<td>-8</td>
<td>UUAUUGGCAGUGUUGGUA</td>
<td>63</td>
</tr>
<tr>
<td>CUACCAACACUGACCAAUAA</td>
<td>-8</td>
<td>UAUUGGCAGUGUUGGUAG</td>
<td>63</td>
</tr>
<tr>
<td>ACCAACACUGACCAUAAC</td>
<td>-8</td>
<td>UUUUUUGGCAGUGUUGG</td>
<td>65</td>
</tr>
<tr>
<td>ACAACACUCCUACCAACU</td>
<td>-8</td>
<td>AGUGUUGGUAGGAGU</td>
<td>74</td>
</tr>
<tr>
<td>ACUCCUACCAACACUGACC</td>
<td>-7</td>
<td>GGUCAGUGUUGGUAGAG</td>
<td>62</td>
</tr>
<tr>
<td>CUCUCAACACUAGCACCAC</td>
<td>-7</td>
<td>AUUGGUCAGUGUUGGUAGG</td>
<td>63</td>
</tr>
<tr>
<td>UCCUACCAACACUGACCAAA</td>
<td>-7</td>
<td>UUGGUCAGUGUUGGUAGGA</td>
<td>63</td>
</tr>
<tr>
<td>AAACUCCUACCAACACUGA</td>
<td>-6</td>
<td>UCAGUGUUGGUAGGAGU</td>
<td>64</td>
</tr>
<tr>
<td>CCAACACUGACCAUAAC</td>
<td>-6</td>
<td>UUUUUUGGCAGUGUUGG</td>
<td>66</td>
</tr>
<tr>
<td>CAAACUCUCCUACCAACUG</td>
<td>-6</td>
<td>CAGUGUUGGUAGGAGG</td>
<td>66</td>
</tr>
<tr>
<td>AACUCCUACCAACACUGC</td>
<td>-6</td>
<td>GUCAGUGUUGGUAGGAGU</td>
<td>67</td>
</tr>
<tr>
<td>AACACUGACCAUAAAAA</td>
<td>-6</td>
<td>UUUUUUGUUGGCAGUGG</td>
<td>69</td>
</tr>
<tr>
<td>CAACACUGACCAUAAAAAA</td>
<td>-6</td>
<td>UUUUUUGUUGGCAGUGG</td>
<td>70</td>
</tr>
<tr>
<td>GCUACAAAAACAGCXXXX</td>
<td>-5</td>
<td>AAGCUUGUUGGUGGUGGAGC</td>
<td>60</td>
</tr>
<tr>
<td>GGCUACAAAAACAGCAAA</td>
<td>-5</td>
<td>AAGCUUGUUGGUGGUGGAGCC</td>
<td>60</td>
</tr>
<tr>
<td>ACACUGACCAUAAAAA</td>
<td>-5</td>
<td>UUUUUUGUUGGCAGUGG</td>
<td>65</td>
</tr>
</tbody>
</table>
The IFN-α score threshold for a Group B siRNA is

\[0.6075 \times n - 9.9484\]

\((n = \text{length of the ssRNA oligonucleotide and } n > 13)\) according to the "addition method".

Both the sense and the antisense strands of an siRNA have to have an IFN-α score below the threshold for the siRNA to be assigned to Group B. In order to maximize the chance of identifying at least one siRNA with optimal gene silencing activity but minimal immunostimulatory activity, at least ten Group B siRNA duplexes should be identified for a given target mRNA. Should this condition not be met, the threshold IFN-α score is increased by 1 in a stepwise manner until ten Group B siRNA are identified.

The IFN-α score threshold for Group B 19mer siRNA duplexes is 1.5941.

None of the 833 potential hCyPB siRNA have an IFN-α score of lower than 1.5491 for both strands. When the threshold was increased to 2.5491, one siRNA is identified. In
order to identify at least ten siRNA duplexes, the threshold IFN-α score has to be increased by 3 to 4.5491. The Group B siRNA thus identified are listed in Table 10:

<table>
<thead>
<tr>
<th>Sense strand</th>
<th>IFN-α score</th>
<th>(5'-&gt; 3')</th>
<th>Antisense strand</th>
<th>IFN-α score</th>
<th>(5'-&gt; 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAGAUCGAGGGAGAAGGC</td>
<td>4</td>
<td>GCUUCUCCACCUCGAUCUU</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAGAUCGAGGGAGAAGGCC</td>
<td>4</td>
<td>GGCUUCUCCACCUCGAUCU</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCGCCGCGCAUCGCGGGG</td>
<td>4</td>
<td>CCCGCGAUGAGGGCGGCGG</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCUUCUGCGGCGGAUGAGA</td>
<td>2</td>
<td>UCUCAUCGGCGCGAGA</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGCGGCCCCUCAUCGCGGGG</td>
<td>4</td>
<td>CCCGCGAUGAGGGCGGCG</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CUUCCUGCUCCUGCGGGA</td>
<td>4</td>
<td>UCCCCGAGCGAGGAAG</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAGCGUUCUCCCGAGA</td>
<td>2</td>
<td>UCUCAUCGGGAAGCGUC</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCCGCCGCGCAUCGCGG</td>
<td>4</td>
<td>CCCGCGAUGAGGGCGGCGC</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGACAAGCUGAGGUGGAGA</td>
<td>4</td>
<td>UCUCCACUCGACUCUUCG</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCUCGCGUCUCGCGC</td>
<td>4</td>
<td>CCCGCGAGCGAGGAAGA</td>
<td>-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UGCCGCGCCCGGCGGCGG</td>
<td>4</td>
<td>CGCGAUGAGGGCGGCGGGA</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Example 12.** Rational identification of a potent 4mer ssRNA motif with maximal IFN-α induction in PBMC

Previous experiments have shown that a minimal length of 19 bases is required for maximal IFN-α induction by ssRNA oligonucleotides. Since poly A oligonucleotides proved to be inactive in terms of IFN-α induction, we decided to analyze sequence requirements for IFN-α induction in PBMC by placing putative motifs into the center of poly A RNA oligonucleotides. RNA9.2sense (5'-AGCUUAACCUGUCCUUCA-3') was used as an established positive control. We started by analyzing the impact of a single nucleotide exchange in the center of a poly A RNA oligonucleotide.

As expected, little IFN-α induction could be elicited by these oligonucleotides, yet a consistent induction was seen for the ssRNA oligonucleotide containing a Uracil (U) in the center of the poly-A chain (Figure 9A).

Based on these data, a panel of ssRNA oligonucleotides was designed that contained a U in the center of a 19mer poly A oligonucleotide including all possible single base exchanges.
permutations to either the 5' or the 3' end (Figure 9B). Compared to the 1mer motifs, a considerable increase in IFN-α induction by the tested 2mer motifs could be seen. A wide distribution with an approximately 100-fold difference between the weakest and the strongest motif was observed: 5'-CU-3', 0.24% of control vs. 5'-GU-3', 24.63% of control. Among all 2mer motifs tested, 5'-GU-3' turned out to be the most potent motif with a mean IFN-α induction of 24.63% of the control oligonucleotide 9.2 sense.

Following these results, oligonucleotides were designed that contained the 5'-GU-3' motif in the center of a 19mer poly A oligonucleotide while again all possible single base permutations to either the 5' or the 3' end of the central motif were tested (Figure 9C). Compared to 5'-GU-3', an almost 6-fold increase in IFN-α induction was seen for the most potent oligonucleotide that minimally contained the sequence 5'-GUC-3' as the central motif. This oligonucleotide exceeded the control oligonucleotide by 1.43-fold in terms of IFN-α induction. A considerable amount of IFN-α, yet significantly less, was induced by the second best motif combination 5'-GUU-3' (88% of control).

Based on these data, we again designed oligonucleotides that contained the identified minimal 3mer motif 5'-GUC-3' with single base permutations to either the 5' or the 3' end. While the transition from 1mer motifs to 2mer motifs and from 2mer motifs to 3mer motifs had resulted in a strong increase in IFN-α induction, no additional enhancement in IFN-α induction was seen with the elongation of the central motif to a 4mer motif (Figure 9D). Nevertheless, these data indicated that A was required at the 3' end of the 5'-GUC-3' motif for maximal IFN-α induction. Placing either C or U at the 3' end of the 5'-GUC-3' motif resulted in a reduction of approximately one third in IFN-α induction, whereas the addition of G resulted in an almost two third decrease in IFN-α production. No further increase in IFN-α induction was seen when the position to the 5'end of the 5'-GUC-3' motif was permutated. While changing the A to G resulted in a slight, yet not significant decrease in IFN-α induction, a decrease of approximately one third was obtained when either U or C were positioned at the 5' end. Altogether these data identified the 4mer sequence 5'-GUCA-3' as a potent motif for the induction of IFN-α in PBMC. Additional modification of the 3'end by single base permutations did not result in an increase in IFN-α induction (Figure 9E), thereby indicating the maximal requirement of a 4mer motif for potent IFN-α induction.
Example 13. The effect of position and number of 4mer motifs on immunostimulatory activity

The position of the 4mer motif within the poly A backbone had little impact on the immunostimulatory activity of the respective oligonucleotide (Figure 7). Only a slight decrease (12.6 %) in IFN-α induction was seen, when the 4mer motif was moved to 5’ end of the oligonucleotide, while positioning of the 4mer motif at the 3’ end resulted in a decrease of 28.8% in IFN-α induction. Moving the motif two nucleotides to the 5’ end from the center position resulted in an almost identical IFN-α inducing activity.

Moreover, no inhibitory effect was seen, when non-stimulatory motifs were introduced into ssRNA oligonucleotides that contained the 4mer motif 5’-GUCA-3’ (data not shown). More importantly, when additional 5’-GUCA-3’ motifs were introduced into a ssRNA oligonucleotide, no further increase in IFN-α induction could be observed (Figure 10).

Example 14. Identification of G - U - Pyrimidine as the optimal IFN-α inducing RNA motif using a ssRNA oligonucleotide library encompassing all possible 4mer motifs

The approach of gradually refining a stimulatory motif by permutation of the adjoining bases is based on the assumption that the exact succession of specific bases is critical for the stimulatory capacity of the sequence. The toleration of per se "non-stimulatory inserts" into an active sequence cannot be accounted for by this experimental setup. Moreover, this approach would miss stimulatory motifs if the sequential combination of several by itself non-stimulatory bases could synergize to render a potent motif. To test for these possibilities a library of ssRNA oligonucleotides was designed that encompassed all possible 4mer motifs in the centre of a poly A oligonucleotide. In view of the fact that flanking A residues can be part of a 4mer RNA sequence motif, only 193 ssRNA oligonucleotides (Table 1) were needed to cover all 256 possible 4mer motifs.

All 193 ssRNA oligonucleotides were tested on PBMC from six individual healthy donors. RNA9.2sense (5’-AGCUUAACCUGUCCUUCAA-3 ’) was included as a positive control on all cell culture plates and was used as a reference for the oligonucleotides tested (RNA9.2sense = 1, all data are expressed as fold values). As typically performed for large data sets such as gene array data, a global normalization to the mean was
performed for each individual donor by subtracting the mean of all data from a particular donor from the individual raw data. This allowed to control for the observed inter individual variability between the donors and made it possible to summarize all donors as mean values ± SEM. Thus due to the normalization, a negative value was obtained for all oligonucleotides that were below the immunostimulatory activity of the mean of all oligonucleotides, whereas for all oligonucleotides that were above the immunostimulatory activity of the mean a positive value was obtained. A colored output was chosen to give an overview on all obtained data in one graph in ascending order (Figure 11A), while the corresponding mean values are depicted next to it (Figure 11B). A complete list of all data with the respective sequence information is depicted in Figure 3. Confirming the validity of our prior approach, the 4mer motif 5'-GUCA-3' turned out to be the second highest hit in the obtained data set, whereas only the motif 5'-GUUC-3' turned out to be more active in terms of IFN-α induction.

To systematically identify motifs or patterns that were associated with high or low IFN-α induction, a statistical analysis was performed by analyzing the occurrence of all possible 3mer motifs within the tested oligonucleotide library. For all 3mer motifs the mean level of IFN-α induction was calculated by grouping all oligonucleotides that contained the respective 3mer motif.

For example the 3mer motif 5'-GUC-3' was contained in ssRNA oligonucleotides ANP 35, 83, 131, 137, 138, 139 and 179 with respective IFN-α induction levels of 1.33, 0.68, 0.93, 0.79, 0.44, 0.84 and 0.73. The mean IFN-α induction level of the 3mer motif 5'-GUC-3' was thus calculated to be 0.82 with a standard error of mean of 0.10.

3mer motifs that were gapped by one nucleotide between either the first and the second nucleotide position or the second and third nucleotide position were also included in this analysis. A two-tailed T-Test was used to identify motifs that were either significantly higher or lower in IFN-α induction than the particular motif analyzed.

For ungapped 3mer motifs, the highest mean level of IFN-α induction was obtained for the motif 5'-GUU-3' (0.87) followed by the motif 5'-GUC-3' (0.82). Within 3mer motifs that contained a nucleotide gap between the first and second position 5'-GNUC-3' (0.87) and 5'-GNUU-3' (0.72) were the two highest hits, where N represents any one nucleotide A, G, U or C. Correspondingly 5'-GUNU-3' (0.83) and 5'-GUNC-3' (0.72) were the two
most potent 3mer motifs within the group of 3mer motifs, which had a nucleotide gap between the second and the third base position (Figure 12). A detailed list of all motifs and the respective mean levels of IFN-α induction is given in Table 12.

Among the top 5% of the tested ssRNA oligonucleotide library, all oligonucleotides contained at least one of the above-mentioned most potent motifs and this was also true for 79% of all top 10% oligonucleotides (Figure 13). The mean level of IFN-α induction for all oligonucleotides that contained at least one of the above motifs was calculated to be $0.72 \pm 0.09$ compared to $-0.09 \pm 0.01$ for the rest of the oligonucleotides. Altogether this analysis was able to identify the motif G - U - Pyrimidine with a one-gap tolerance between either the first and the second or the second and the third position as a potent motif for RNA-mediated IFN-α induction in the human system.

**Example 15.** Comparison of immunostimulatory activity

Comparing the motif 5'-GUUC-3' on a poly A backbone to previously published ssRNA oligonucleotides with high IFN-α inducing activity revealed an almost 1.4 fold higher level of IFN-α induction than 9.2sense 5'-AGCUUAACCUGCUCCUCAA-3' (Hornung et al. Nat Med 11:263:270), 1.5 fold higher level than RNA40 5'-GCCGUGUGUGUGACUC-3' (Heil et al. Science 303:1526-1529) and a more than 2.5 fold higher activity than β-Gal control sense 5'-UGAUGUGUUAGUCGCU-3' (Judge et al. Nat Biotech 23:457-462) (Figure 14).

**Example 16.** Using the obtained motif information to predict low or high IFN-α inducing ssRNA oligonucleotides

An analysis of published ssRNA oligonucleotides that were described to induce IFN-α indicated a good correlation with our motif information. To systematically predict the IFN-α inducing activity of a ssRNA oligonucleotide, an algorithm was established based on the occurrence of 3mer motifs 5'-NNN-3'. Since above data had indicated that the major IFN-α inducing activity of a ssRNA oligonucleotide was independent of the position of the stimulatory motif and the presence of additional stimulatory or inhibitory motifs, an algorithm was developed that predicted the IFN-α inducting activity by accounting for the
highest stimulatory motif within the oligonucleotide independent of its position. Only motifs that had been shown to significantly correlate with high or low IFN-α inducing activity were included in the algorithm: 18 ungapped 3mer motifs (5’-NNN-3’), indicated by “*” or “**” in Table 12A. Thus for a given oligonucleotide, a predicted IFN-α inducing activity was calculated by analyzing the occurrence of all 3mer motifs within this oligonucleotide and subsequently by assigning the highest obtained mean IFN-α induction level to this respective oligonucleotide.

Applying this algorithm (i.e., the "simplified method") to our own data set resulted in a correlation coefficient of 0.80 for the comparison of the predicted and the measured data (Figure 15).

The predictive quality of this algorithm to define low IFN-α inducing ssRNA oligonucleotides was then tested on the 193 oligo data set. Low inducing oligonucleotides were predefined by an IFN-α induction level below the mean of all oligonucleotides. Various threshold levels for the prediction algorithm were tested for both for the positive predictive value and the sensitivity to identify ssRNA oligonucleotides below the mean IFN-α of all oligonucleotides (Figure 16). A high positive predictive value and a yet high sensitivity was obtained when the threshold of the prediction algorithm was set at 0.11 (Figure 16B, upper right panel). 118 oligonucleotides had a calculated predicted IFN-α induction level below the threshold of 0.11 and 117 of these oligonucleotides had the expected measured IFN-α level below 0 (positive predictive value of 99.15%). Only 28 oligonucleotides of a total of 145 oligonucleotides were not detected using the prediction (sensitivity of 80.69%). Using the prediction algorithm with the threshold of 0.11 therefore allowed to predict a large percentage of oligonucleotides that would be below the desired level of 0 with high accuracy.

Applying this algorithm to more complex oligonucleotides resulted in a considerable lower percentage of oligonucleotides that met the above criteria. Approximately 10% of a random pool of 19mer oligonucleotides fell below the threshold of 0.11 when analyzed using the IFN-α prediction algorithm (data not shown). When a random pool of 19mer duplexes was analyzed, approximately 1% of all duplexes were comprised of single stranded RNA oligonucleotides that were both below the threshold of 0.11.
**Example 17.** Preparing siRNA with high or low immunostimulatory activity

To address the applicability of this algorithm for the identification of non-stimulatory RNA oligonucleotides, a panel of siRNA duplexes was designed to target the mRNA of human TLR9. From a total pool of 3850 siRNA duplexes that were considered, 116 could be identified (3.01%) that had a calculated predicted IFN-α induction below the threshold of 0.11 according to the "simplified method". These 116 predicted siRNA duplexes were distributed over 21 different target sites within the mRNA, whereas seven major sites could be identified that could be targeted by more than five consecutive siRNA duplexes. Three different siRNA duplexes were chosen to target TLR9 using the above described algorithm with putatively low IFN-α induction (TLR9_271, TLR9_1122 and TLR9_1949), whereas three different siRNA duplexes were picked that contained at least one strand with predicted high IFN-α induction (TLR9_1019, TLR9_1302 and TLR9_1949). Altogether eight ssRNA oligonucleotides with presumably low IFN-α induction (TLR9_271 sense, TLR9_271 antisense, TLR9_1019 sense, TLR9_1122 sense, TLR9_1122 antisense, TLR9_1302 antisense, TLR9_1949 sense, TLR9_1949 antisense) and four ssRNA oligonucleotides with high IFN-α induction (TLR9_iO19 antisense, TLR9_1302 sense, TLR9_1500 sense and TLR9_1500 antisense) were contained in this selection (Figure 17).

The functionality of these siRNA duplexes in terms of posttranscriptional gene silencing was assessed by analyzing the knock down activity of TLR9 expression (Figure 18). Two siRNA duplexes with good knock down activity could be identified within both groups of siRNA duplexes.

When analyzing the IFN-α induction of both the single stranded components and the respective siRNA duplexes, a high accuracy of the IFN-α prediction algorithm could be seen. Of all eight ssRNA oligonucleotides predicted to be low in IFN-α induction, seven oligonucleotides (TLR9_271 antisense, TLR9_1019 sense, TLR9_1122 sense, TLR9_1122 antisense, TLR9_1302 antisense, TLR9_1949 sense, TLR9_1949 antisense) showed negligible to absent IFN-α induction, whereas one oligonucleotide (TLR9_271 sense) showed minimal IFN-α induction. This was also true for the respective siRNA duplexes: TLR9_1122 and TLR9_1949 duplexes showed negligible to absent IFN-α induction, and TLR9_271 duplex showed minimal IFN-α induction. Likewise, all
ssRNA oligonucleotides predicted to be high in IFN-α induction showed a strong IFN-α response (TLR9_1019 antisense, TLR9_1302 sense, TLR9_1500 sense and TLR9_1500 antisense). As described previously, within a siRNA duplex, the strong induction of IFN-α by either one component dictated the immunostimulatory activity of the whole siRNA duplex. TLR9_1019, TLR9_1032 and TLR9_1500 duplexes all showed high IFN-α induction.

Altogether these results indicated that using a motif-based algorithm we are able to rationally design both stimulatory and non-stimulatory functional siRNA duplexes.

**Example 18.** Systematic and automated identification of siRNA with desired immunostimulatory activity.

Based on the algorithm described in example 17 (i.e., the "simplified method"), a computer program was written that applies the algorithm to all possible siRNA duplexes targeting all human RNA transcripts (50421 as of 09/2006) as published by the National Center for Biotechnology Information (NCBI). Each entry into the NCBI database (ftp://ftp.ncbi.nih.gov/refseq/H_sapiens/mRNA_Prot/human.ma.fna.gz) of all listed human RNA transcripts was analyzed the following way: A list of all possible 19mer siRNA duplexes targeting a given RNA transcript was generated. Of all siRNA duplexes the IFN-α induction of both the sense and the antisense strand was predicted using the method described in example 17. The obtained data is stored in a database (CD-ROM) and can be retrieved by a search engine. Using the search interface, the user can pick the transcript of interest (alphabetical index of all RNA transcripts targeted by siRNAs) and then adjust the level of threshold to identify siRNA duplexes that are of either low, intermediate or high in immunostimulatory activity (Fig. 19A). For example, using the threshold of 0.11 as described in example 17, a set of siRNA duplexes was identified for Homo sapiens vascular endothelial growth factor (VEGF) transcript variant 1 mRNA (NM_001025366.1) with low immunostimulatory activity for both the sense and the antisense strand (Figure 19B).

**Tablei:** ssRNA oligonucleotides containing all possible 4mer motifs on the poly a backbone.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
</table>

...
<table>
<thead>
<tr>
<th>ANP-Oligo</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>AAAAAAAAAAAAAAAAAAAAAAAAAAA</td>
</tr>
<tr>
<td>002</td>
<td>AAAAAAAAACAAAAA</td>
</tr>
<tr>
<td>003</td>
<td>AAAAAAAAAAAAAAAA</td>
</tr>
<tr>
<td>004</td>
<td>AAAAAAAAAUAUAAAAAAAAAAAAA</td>
</tr>
<tr>
<td>005</td>
<td>AAAAAAAAAACAAAAAAA</td>
</tr>
<tr>
<td>006</td>
<td>AAAAAAAAACGAAAAAAA</td>
</tr>
<tr>
<td>007</td>
<td>AAAAAAAAACUAAAAAAAAAAAAA</td>
</tr>
<tr>
<td>008</td>
<td>AAAAAAAAAAGCAAAAAAAAAAAAA</td>
</tr>
<tr>
<td>009</td>
<td>AAAAAAAAAAGGAAAAAAA</td>
</tr>
<tr>
<td>010</td>
<td>AAAAAAAAAAGUAAAAAAAAAAAAA</td>
</tr>
<tr>
<td>011</td>
<td>AAAAAAAAAACUAEEEEEEEEEEEEEE</td>
</tr>
<tr>
<td>012</td>
<td>AAAAAAAAAAGUAAAAAAA</td>
</tr>
<tr>
<td>013</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>014</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>015</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>016</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>017</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>018</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>019</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>020</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>021</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>022</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>023</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>024</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>025</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>026</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>027</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>028</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>029</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>030</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>031</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>032</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>033</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>034</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>035</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
<tr>
<td>036</td>
<td>AAAAAAAAAACUAAAAAAA</td>
</tr>
</tbody>
</table>
ANP-Oligo 037  AAAAAAAAGUUAAAAAAA
ANP-Oligo 038  AAAAAAAAUCAAAAAA
ANP-Oligo 039  AAAAAAAAUAGAAAAAAA
ANP-Oligo 040  AAAAAAAAUUAUAAAAAAA
ANP-Oligo 041  AAAAAAAAUCCAAAAAAA
ANP-Oligo 042  AAAAAAAAUCGAAAAAAA
ANP-Oligo 043  AAAAAAAAUCUAAAAAAA
ANP-Oligo 044  AAAAAAAAUGCAAAAAAAA
ANP-Oligo 045  AAAAAAAAUGGAAAAAAA
ANP-Oligo 046  AAAAAAAAUGUAAAAAAA
ANP-Oligo 047  AAAAAAAAUCUAAAAAAA
ANP-Oligo 048  AAAAAAAAUAUGAAAAAAA
ANP-Oligo 049  AAAAAAAAUAUUAAAAAAA
ANP-Oligo 050  AAAAAAACACAAAAAAA
ANP-Oligo 051  AAAAAAACAGAAAAAAA
ANP-Oligo 052  AAAAAAACAUAAAAAAA
ANP-Oligo 053  AAAAAAACACUAAAAAAA
ANP-Oligo 054  AAAAAAACACGAAAAAAA
ANP-Oligo 055  AAAAAAACACUAUUAAAAAA
ANP-Oligo 056  AAAAAAACAGCAAAAAAAA
ANP-Oligo 057  AAAAAAACAGGAAAAAAA
ANP-Oligo 058  AAAAAAACAGUAAAAAAA
ANP-Oligo 059  AAAAAAACAUCAUUAAAAAA
ANP-Oligo 060  AAAAAAACAUCAUUAAAAAA
ANP-Oligo 061  AAAAAAACAUCAUUAAAAAA
ANP-Oligo 062  AAAAAAACACAAAAAAA
ANP-Oligo 063  AAAAAAACACAAAAAAA
ANP-Oligo 064  AAAAAAACACAAAAAAA
ANP-Oligo 065  AAAAAAACACAAAAAAA
ANP-Oligo 066  AAAAAAACACAAAAAAA
ANP-Oligo 067  AAAAAAACACAAAAAAA
ANP-Oligo 068  AAAAAAACACAAAAAAA
ANP-Oligo 069  AAAAAAACACAAAAAAA
ANP-Oligo 070  AAAAAAACACAAAAAAA
ANP-Oligo 071  AAAAAAACACAAAAAAA
ANP-Oligo 072  AAAAAAACACAAAAAAA
ANP-Oligo 073 AAAAAAACCUUAAAAA
ANP-Oligo 074 AAAAAAACGACAAAAAA
ANP-Oligo 075 AAAAAAACGAGAAAAAA
ANP-Oligo 076 AAAAAAACGAUAAAAAA
ANP-Oligo 077 AAAAAAACGCCAAAAAA
ANP-Oligo 078 AAAAAAACGCGAAAAAA
ANP-Oligo 079 AAAAAAACGCUAAAAAA
ANP-Oligo 080 AAAAAAACGGCAAAAAAA
ANP-Oligo 081 AAAAAAACGGGAAAAAA
ANP-Oligo 082 AAAAAAACGGUAAAAAA
ANP-Oligo 083 AAAAAAACGUCAAAAAAA
ANP-Oligo 084 AAAAAAACGUGAAAAAA
ANP-Oligo 085 AAAAAAACGUAAAAAA
ANP-Oligo 086 AAAAAAACGUUAAAAAA
ANP-Oligo 087 AAAAAAACUACAAAAAA
ANP-Oligo 088 AAAAAAACUAGAAAAAA
ANP-Oligo 089 AAAAAAACUAUAAAAAA
ANP-Oligo 090 AAAAAAACUCCAAAAAA
ANP-Oligo 091 AAAAAAACUCGAAAAAA
ANP-Oligo 092 AAAAAAACUGCAAAAAAA
ANP-Oligo 093 AAAAAAACUCGAAAAAA
ANP-Oligo 094 AAAAAAACUGUAAAAAA
ANP-Oligo 095 AAAAAAACUGUCAAAAAAA
ANP-Oligo 096 AAAAAAACUGUAAAAAA
ANP-Oligo 097 AAAAAAACUGUAAAAAA
ANP-Oligo 098 AAAAAAAGAAGAAAAA
ANP-Oligo 099 AAAAAAAGAGAAAAA
ANP-Oligo 100 AAAAAAAGAGAAAAA
ANP-Oligo 101 AAAAAAAGAGAAAAA
ANP-Oligo 102 AAAAAAAGAGAAAAA
ANP-Oligo 103 AAAAAAAGAGAAAAA
ANP-Oligo 104 AAAAAAAGAGAAAAA
ANP-Oligo 105 AAAAAAAGAGAAAAA
ANP-Oligo 106 AAAAAAAGAGAAAAA
ANP-Oligo 107 AAAAAAAGAGAAAAA
ANP-Oligo 108 AAAAAAAGAGAAAAA
ANP-Oligo 109 AAAAAAAGAUUAAAAAAA
ANP-Oligo 110 AAAAAAAGCACAABAAAAAA
ANP-Oligo 111 AAAAAAAGCAGAAAAAAA
ANP-Oligo 112 AAAAAAAGCAUAABAABA
ANP-Oligo 113 AAAAAAAGCCEAAABAAA
ANP-Oligo 114 AAAAAAAGCCGAAAAAAA
ANP-Oligo 115 AAAAAAAGCCUAABAABA
ANP-Oligo 116 AAAAAAAGCGCAAABAAA
ANP-Oligo 117 AAAAAAAGCGGAABAAAA
ANP-Oligo 118 AAAAAAAGCGUAABAAAA
ANP-Oligo 119 AAAAAAAGCUCAABAAAA
ANP-Oligo 120 AAAAAAAGCUUAABAABA
ANP-Oligo 121 AAAAAAAGCUUAABAAAA
ANP-Oligo 122 AAAAAAAGGACABAAAAA
ANP-Oligo 123 AAAAAAAGGAGAAAABA
ANP-Oligo 124 AAAAAAAGGAUAABAAAA
ANP-Oligo 125 AAAAAAAGGCCABAAAAA
ANP-Oligo 126 AAAAAAAGGCGAAAABA
ANP-Oligo 127 AAAAAAAGGCUAABAAAA
ANP-Oligo 128 AAAAAAAGGGCABAAAAA
ANP-Oligo 129 AAAAAAAGGGGAAAABA
ANP-Oligo 130 AAAAAAAGGGUAABAAAA
ANP-Oligo 131 AAAAAAAGGUCABAABA
ANP-Oligo 132 AAAAAAAGGUGAAABAAA
ANP-Oligo 133 AAAAAAAGGUAAABAAAA
ANP-Oligo 134 AAAAAAAGUACABAABA
ANP-Oligo 135 AAAAAAAGUAGAAAABA
ANP-Oligo 136 AAAAAAAGUUAABAABA
ANP-Oligo 137 AAAAAAAGUUCABAAAAB
ANP-Oligo 138 AAAAAAAGUCGAAABAAA
ANP-Oligo 139 AAAAAAAGUCUAABAAAA
ANP-Oligo 140 AAAAAAAGUGCAABAAAAB
ANP-Oligo 141 AAAAAAAGUGGAAAABA
ANP-Oligo 142 AAAAAAAGUGUAABAAAA
ANP-Oligo 143 AAAAAAAGUUCAABAAAA
ANP-Oligo 144 AAAAAAAGUUGAAAAAAA
ANP-Oligo 145       AAAAAAGUUUAAAAAAAAAA
ANP-Oligo 146       AAAAAAAAUACAAAAAA
ANP-Oligo 147       AAAAAAAUAAGAAAAAAA
ANP-Oligo 148       AAAAAAAUAUUAAAAAAA
ANP-Oligo 149       AAAAAAAUAACAAAAAAA
ANP-Oligo 150       AAAAAAAUAACGAAAAAAA
ANP-Oligo 151       AAAAAAAUAACUAAAAAAA
ANP-Oligo 152       AAAAAAAUAGAAAAAAA
ANP-Oligo 153       AAAAAAAUAGGAAAAAAA
ANP-Oligo 154       AAAAAAAUAGUAAAAAAA
ANP-Oligo 155       AAAAAAAUAUCAAAAAAA
ANP-Oligo 156       AAAAAAAUAUGAAAAAAA
ANP-Oligo 157       AAAAAAAUAUJAAAAAAA
ANP-Oligo 158       AAAAAAAUCACAABAAAA
ANP-Oligo 159       AAAAAAAUCAGAAAAAAA
ANP-Oligo 160       AAAAAAAUCAUAAAAAAA
ANP-Oligo 161       AAAAAAAUCCAAAAAAAA
ANP-Oligo 162       AAAAAAAUCCGAAAAAAA
ANP-Oligo 163       AAAAAAAUCCUAAAAAAA
ANP-Oligo 164       AAAAAAAUCGCABAAAA
ANP-Oligo 165       AAAAAAAUCGGAAAAAAA
ANP-Oligo 166       AAAAAAAUCGUAAAAAAA
ANP-Oligo 167       AAAAAAAUCUGAAAAAAA
ANP-Oligo 168       AAAAAAAUCUGAAAAAAA
ANP-Oligo 169       AAAAAAAUCUAAAAAAA
ANP-Oligo 170       AAAAAAAUGACAAAAAAA
ANP-Oligo 171       AAAAAAAUGAGAAAAAAA
ANP-Oligo 172       AAAAAAAUGAUAAAAAAA
ANP-Oligo 173       AAAAAAAUGCCAAAAAAA
ANP-Oligo 174       AAAAAAAUGCGAAAAAAA
ANP-Oligo 175       AAAAAAAUGCUAAAAAAA
ANP-Oligo 176       AAAAAAAUGGCAAAAAAA
ANP-Oligo 177       AAAAAAAUGGGAAAAAAA
ANP-Oligo 178       AAAAAAAUGGUAAAAAAA
ANP-Oligo 179       AAAAAAAUGUCAAAAAAA
ANP-Oligo 180       AAAAAAAUGUGAAAAAAA
ANP-Oligo 181   AAAAAAAUGUUAAAAAAAA
ANP-Oligo 182   AAAAAAAUUACAAAAAAA
ANP-Oligo 183   AAAAAAAUUAGAAAAAAA
ANP-Oligo 184   AAAAAAAUUUAUAAAAAAA
ANP-Oligo 185   AAAAAAAUUUGAAAAAAA
ANP-Oligo 186   AAAAAAAUUUCGAAAAAAA
ANP-Oligo 187   AAAAAAAUUUCUAAAAAAA
ANP-Oligo 188   AAAAAAAAUUGCAAAAAAAA
ANP-Oligo 189   AAAAAAAAUUGGAAAAAAA
ANP-Oligo 190   AAAAAAAAUUGUAAAAAAA
ANP-Oligo 191   AAAAAAAAUUUCAAAAAAAA
ANP-Oligo 192   AAAAAAAAUUUGAAAAAAA
ANP-Oligo 193   AAAAAAAAUUUUAAAAAAA

Table 2: Group 1 ssRNA-Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5'→3'</th>
<th>adjusted IFN-α index</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP-Oligo 018</td>
<td>AAAAAAAACCGAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 020</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 029</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 051</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 053</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 075</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 080</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 128</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 130</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 009</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 017</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 003</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 081</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 113</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo 014</td>
<td>AAAAAAAACGCAAAAAAAA</td>
<td>-0,23</td>
<td>0,03</td>
</tr>
<tr>
<td>ANP-Oligo</td>
<td>Sequence</td>
<td>ΔG</td>
<td>ΔΔG</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>077</td>
<td>AAAAAACGCCAAAAAAAAAAAAAA</td>
<td>-0.23</td>
<td>0.03</td>
</tr>
<tr>
<td>126</td>
<td>AAAAAAGGCGAAAAAAAAAAAAAA</td>
<td>-0.23</td>
<td>0.03</td>
</tr>
<tr>
<td>125</td>
<td>AAAAAAGGCGAAAAAAAAAAAAAA</td>
<td>-0.23</td>
<td>0.03</td>
</tr>
<tr>
<td>050</td>
<td>AAAAAAAACACAAAAAAA</td>
<td>-0.23</td>
<td>0.03</td>
</tr>
<tr>
<td>015</td>
<td>AAAAAAAACACGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>114</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>027</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>117</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>006</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>005</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>069</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>066</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>007</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>060</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>021</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>042</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>086</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>064</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>102</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>068</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>129</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>008</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>002</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>038</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>026</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>116</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>054</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>057</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.22</td>
<td>0.03</td>
</tr>
<tr>
<td>001</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>074</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>033</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>123</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>059</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>110</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>065</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>055</td>
<td>AAAAAAAGGCGGAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>Oligo</td>
<td>Sequence</td>
<td>E-value</td>
<td>p-value</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>ANP-Oligo 122</td>
<td>AAAAAAAGGACAAAAAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>ANP-Oligo 104</td>
<td>AAAAAAAGAGGCAAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>ANP-Oligo 078</td>
<td>AAAAAAACGCGAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>ANP-Oligo 032</td>
<td>AAAAAAAAGGCAAAAAAAA</td>
<td>-0.21</td>
<td>0.04</td>
</tr>
<tr>
<td>ANP-Oligo 030</td>
<td>AAAAAAAAGCGAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>ANP-Oligo 004</td>
<td>AAAAAAAUAUAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>ANP-Oligo 105</td>
<td>AAAAAAAGAGGAAAAAAA</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>ANP-Oligo 161</td>
<td>AAAAAAAUCCCAAAAAAAA</td>
<td>-0.21</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo 061</td>
<td>AAAAAAACAUUAAAAAAA</td>
<td>-0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo 041</td>
<td>AAAAAAAUAUCCAAAAAAA</td>
<td>-0.2</td>
<td>0.03</td>
</tr>
<tr>
<td>ANP-Oligo 063</td>
<td>AAAAAAACAGCAAAAAAAAA</td>
<td>-0.2</td>
<td>0.04</td>
</tr>
<tr>
<td>ANP-Oligo 072</td>
<td>AAAAAAACUGAAAAAAA</td>
<td>-0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo 101</td>
<td>AAAAAAGACAAAAAAA</td>
<td>-0.2</td>
<td>0.04</td>
</tr>
<tr>
<td>ANP-Oligo 056</td>
<td>AAAAAACAGCAAAAAAAA</td>
<td>-0.2</td>
<td>0.04</td>
</tr>
<tr>
<td>ANP-Oligo 162</td>
<td>AAAAAAUCCGAAAAAAA</td>
<td>-0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo 099</td>
<td>AAAAAAAAGAAGAAAAAAA</td>
<td>-0.2</td>
<td>0.05</td>
</tr>
<tr>
<td>ANP-Oligo 111</td>
<td>AAAAAAACGCAAAAAAAA</td>
<td>-0.2</td>
<td>0.04</td>
</tr>
<tr>
<td>ANP-Oligo 011</td>
<td>AAAAAAAAUCAAAAAAAAA</td>
<td>-0.2</td>
<td>0.04</td>
</tr>
<tr>
<td>ANP-Oligo 062</td>
<td>AAAAAAACACAAAAAAA</td>
<td>-0.19</td>
<td>0.04</td>
</tr>
<tr>
<td>ANP-Oligo 016</td>
<td>AAAAAAAACAUAAAAAAA</td>
<td>-0.19</td>
<td>0.03</td>
</tr>
<tr>
<td>ANP-Oligo 177</td>
<td>AAAAAAAUGCAAAAAAA</td>
<td>-0.19</td>
<td>0.03</td>
</tr>
<tr>
<td>ANP-Oligo 019</td>
<td>AAAAAAAACCUAAAAAAA</td>
<td>-0.19</td>
<td>0.03</td>
</tr>
<tr>
<td>ANP-Oligo 098</td>
<td>AAAAAAAGAACAAAAAAAA</td>
<td>-0.19</td>
<td>0.05</td>
</tr>
<tr>
<td>ANP-Oligo 052</td>
<td>AAAAAAACAAUAAAAAAAA</td>
<td>-0.19</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo 089</td>
<td>AAAAAACUCAAAAAAA</td>
<td>-0.18</td>
<td>0.05</td>
</tr>
<tr>
<td>ANP-Oligo 067</td>
<td>AAAAAAACCUAAAAAAA</td>
<td>-0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo 040</td>
<td>AAAAAAAUAUAAAAAAA</td>
<td>-0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo 039</td>
<td>AAAAAAAUAAGAAAAAAA</td>
<td>-0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo 043</td>
<td>AAAAAAAAUCAAAAAAAA</td>
<td>-0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo 073</td>
<td>AAAAAAACCUAAAAAAA</td>
<td>-0.18</td>
<td>0.01</td>
</tr>
<tr>
<td>ANP-Oligo 024</td>
<td>AAAAAAACUGAAAAAAA</td>
<td>-0.17</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo 028</td>
<td>AAAAAAAGAUAAAAAAAA</td>
<td>-0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>ANP-Oligo 103</td>
<td>AAAAAAGACUAAAAAAAA</td>
<td>-0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>ANP-Oligo 091</td>
<td>AAAAAACUCUAAAAAAA</td>
<td>-0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>ANP-Oligo 158</td>
<td>AAAAAAUCACAAAAAAA</td>
<td>-0.17</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo 012</td>
<td>AAAAAAUGAAAAAAA</td>
<td>-0.16</td>
<td>0.02</td>
</tr>
<tr>
<td>ANP-Oligo</td>
<td>Sequence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>163</td>
<td>AAAAAAAUCCUAAAAAAAA</td>
<td>-0.15 0.03</td>
<td></td>
</tr>
<tr>
<td>025</td>
<td>AAAAAAAACUUAAAAAAAA</td>
<td>-0.15 0.02</td>
<td></td>
</tr>
<tr>
<td>112</td>
<td>AAAAAAAAGCAUAAAAAAAA</td>
<td>-0.15 0.01</td>
<td></td>
</tr>
<tr>
<td>070</td>
<td>AAAAAAACCG UAAAAA</td>
<td>-0.14 0.02</td>
<td></td>
</tr>
<tr>
<td>172</td>
<td>AAAAAAAUGAUAAAAAAA</td>
<td>-0.14 0.03</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>AAAAAAAAUACGAAAAAAA</td>
<td>-0.14 0.04</td>
<td></td>
</tr>
<tr>
<td>146</td>
<td>AAAAAAAUAACAAAAA</td>
<td>-0.14 0.03</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>AAAAAAAAGCCUAAAAAAA</td>
<td>-0.14 0.01</td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>AAAAAAAUCAUAAAAAAA</td>
<td>-0.13 0.02</td>
<td></td>
</tr>
<tr>
<td>151</td>
<td>AAAAAAAAUACUAAAAAAA</td>
<td>-0.13 0.03</td>
<td></td>
</tr>
<tr>
<td>013</td>
<td>AAAAAAAAUUAAAAAAA</td>
<td>-0.13 0.03</td>
<td></td>
</tr>
<tr>
<td>090</td>
<td>AAAAAAACUCGAAAAAAA</td>
<td>-0.13 0.03</td>
<td></td>
</tr>
<tr>
<td>048</td>
<td>AAAAAAAAUUGAAAAAAA</td>
<td>-0.13 0.02</td>
<td></td>
</tr>
<tr>
<td>092</td>
<td>AAAAAAAACUGCAAAAAAA</td>
<td>-0.1 0.02</td>
<td></td>
</tr>
<tr>
<td>093</td>
<td>AAAAAAAACUGGAAAAAAA</td>
<td>-0.13 0.03</td>
<td></td>
</tr>
<tr>
<td>031</td>
<td>AAAAAAAAGCUAAAAAAA</td>
<td>-0.13 0.02</td>
<td></td>
</tr>
<tr>
<td>157</td>
<td>AAAAAAAUAUUAUSAAAAA</td>
<td>-0.12 0.02</td>
<td></td>
</tr>
<tr>
<td>108</td>
<td>AAAAAAAAGAUGAAAAAAA</td>
<td>-0.12 0.02</td>
<td></td>
</tr>
<tr>
<td>076</td>
<td>AAAAAAACUGAUAUSAAAA</td>
<td>-0.12 0.03</td>
<td></td>
</tr>
<tr>
<td>165</td>
<td>AAAAAAAUCGGAUSAAAAA</td>
<td>-0.1 0.03</td>
<td></td>
</tr>
<tr>
<td>087</td>
<td>AAAAAACUCGGAAUSAAAAA</td>
<td>-0.1 0.03</td>
<td></td>
</tr>
<tr>
<td>109</td>
<td>AAAAAAAAGAUPUUSAAAAA</td>
<td>-0.1 0.01</td>
<td></td>
</tr>
<tr>
<td>183</td>
<td>AAAAAAAUAGGUAUSAAAAA</td>
<td>-0.1 0.05</td>
<td></td>
</tr>
<tr>
<td>127</td>
<td>AAAAAAAAGGCUUSAAAAAA</td>
<td>-0.1 0.03</td>
<td></td>
</tr>
<tr>
<td>079</td>
<td>AAAAAACGCUUSAAAAAA</td>
<td>-0.1 0.02</td>
<td></td>
</tr>
<tr>
<td>088</td>
<td>AAAAAACUAUSAAAAAAA</td>
<td>-0.1 0.03</td>
<td></td>
</tr>
<tr>
<td>159</td>
<td>AAAAAUACGUAUSAAAAAA</td>
<td>-0.1 0.05</td>
<td></td>
</tr>
<tr>
<td>152</td>
<td>AAAAAAAUAGCGUSAAAAAA</td>
<td>-0.1 0.04</td>
<td></td>
</tr>
<tr>
<td>084</td>
<td>AAAAAACGUGAAUSAAAAA</td>
<td>-0.1 0.03</td>
<td></td>
</tr>
<tr>
<td>184</td>
<td>AAAAAAAUUAUSAAAAAA</td>
<td>-0.1 0.03</td>
<td></td>
</tr>
<tr>
<td>175</td>
<td>AAAAAAAUGCUSAAAAAAA</td>
<td>-0.1 0.03</td>
<td></td>
</tr>
<tr>
<td>164</td>
<td>AAAAAAAUCGCAUSAAAAAA</td>
<td>-0.09 0.04</td>
<td></td>
</tr>
<tr>
<td>149</td>
<td>AAAAAAAUACUUSAAAAAA</td>
<td>-0.09 0.06</td>
<td></td>
</tr>
<tr>
<td>185</td>
<td>AAAAAAAUUCUUSAAAAAA</td>
<td>-0.08 0.05</td>
<td></td>
</tr>
<tr>
<td>044</td>
<td>AAAAAAAUUGCGUSAAAAAA</td>
<td>-0.08 0.03</td>
<td></td>
</tr>
<tr>
<td>022</td>
<td>AAAAAACGUGUSAAAAAA</td>
<td>-0.08 0.02</td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Group 2 ssRNA-Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5' → 3'</th>
<th>adjusted</th>
<th>SEM</th>
<th>IFN-α index</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP-Oligo 071</td>
<td>AAAAAAACCUCAAAAAAAA</td>
<td>0</td>
<td>0,05</td>
<td></td>
</tr>
<tr>
<td>ANP-Oligo 182</td>
<td>AAAAAAAAUUACAAAAAAA</td>
<td>0,01</td>
<td>0,1</td>
<td></td>
</tr>
<tr>
<td>ANP-Oligo 096</td>
<td>AAAAAAACUUGAAAAAAA</td>
<td>0,02</td>
<td>0,06</td>
<td></td>
</tr>
<tr>
<td>ANP-Oligo 085</td>
<td>AAAAAACGUUUAAAAAAA</td>
<td>0,02</td>
<td>0,04</td>
<td></td>
</tr>
<tr>
<td>ANP-Oligo 176</td>
<td>AAAAAAAGGCUAAAAAAA</td>
<td>0,04</td>
<td>0,04</td>
<td></td>
</tr>
<tr>
<td>ANP-Oligo 187</td>
<td>AAAAAAAUCUGAAAAAAA</td>
<td>0,05</td>
<td>0,04</td>
<td></td>
</tr>
<tr>
<td>ANP-Oligo</td>
<td>Sequence</td>
<td>Efficacy</td>
<td>Selectivity</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------</td>
<td>----------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>166</td>
<td>AAAAAAAUCGUAAAAAAAAAA</td>
<td>0.07</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>023</td>
<td>AAAAAAAACUCAAAAAA</td>
<td>0.08</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>193</td>
<td>AAAAAAAAUUUAAA</td>
<td>0.13</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>188</td>
<td>AAAAAAAUUGCAAAAAA</td>
<td>0.14</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>132</td>
<td>AAAAAAAGGUGAAAAAA</td>
<td>0.18</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>106</td>
<td>AAAAAAAAGAG UAAAAAAAA</td>
<td>0.21</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>095</td>
<td>AAAAAAAACUUCAAAAAA</td>
<td>0.22</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>186</td>
<td>AAAAAAAAU UCGAAAAAAA</td>
<td>0.22</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>058</td>
<td>AAAAAAACAG UAAAAAAAA</td>
<td>0.22</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>167</td>
<td>AAAAAAUCUCAAAAAAA</td>
<td>0.23</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>189</td>
<td>AAAAAAAAU UGGAAAAAAA</td>
<td>0.24</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>047</td>
<td>AAAAAAAAUUCAAAAAA</td>
<td>0.27</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>AAAAAAGUAGAAAAAAA</td>
<td>0.3</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>154</td>
<td>AAAAAAAUAGUAAAAAAA</td>
<td>0.32</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>AAAAAAAGUAUAAAAAAA</td>
<td>0.36</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>134</td>
<td>AAAAAAAGUACAAAAAAA</td>
<td>0.37</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>AAAAAAG UGCAAAAAAA</td>
<td>0.38</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>141</td>
<td>AAAAAAGUGGAAAAAAA</td>
<td>0.39</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>178</td>
<td>AAAAAAUGGUAAAAAAA</td>
<td>0.41</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>192</td>
<td>AAAAAAUUUGAAAAAAA</td>
<td>0.42</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>AAAAAAGUCGAAAAAAA</td>
<td>0.44</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>034</td>
<td>AAAAAAAGGUAAAAAAA</td>
<td>0.46</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>AAAAAAUGUGAAAAAAA</td>
<td>0.46</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>191</td>
<td>AAAAAAUUUCAAAAAAAA</td>
<td>0.54</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>046</td>
<td>AAAAAAAUGUAAAAAAA</td>
<td>0.59</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>037</td>
<td>AAAAAAAAGUUAAAAAAA</td>
<td>0.62</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>181</td>
<td>AAAAAAUGUUAAAAAAA</td>
<td>0.64</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>083</td>
<td>AAAAAAAGCUAAAAAAA</td>
<td>0.68</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>094</td>
<td>AAAAAAACUGUAAAAAAA</td>
<td>0.73</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>179</td>
<td>AAAAAAUGUCAAAAAAA</td>
<td>0.73</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>190</td>
<td>AAAAAAUGUAAAAAAA</td>
<td>0.74</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>121</td>
<td>AAAAAAGCUUAAAAAAA</td>
<td>0.77</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>137</td>
<td>AAAAAAAGUCCA AAAAAA</td>
<td>0.79</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>139</td>
<td>AAAAAAAGUCAAAAAAA</td>
<td>0.84</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>131</td>
<td>AAAAAAGGUCAAAAAAAA</td>
<td>0.93</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>142</td>
<td>AAAAAAGUGUAAAAAAA</td>
<td>0.96</td>
<td>0.08</td>
<td></td>
</tr>
</tbody>
</table>
ANP-Oligo 133 AAAAAAAGGUUAAAAAAAAA 1.04 0.13
ANP-Oligo 145 AAAAAAAGUUAAAAAAAAAA 1.17 0.08
ANP-Oligo 144 AAAAAAAGGUUGAAAAAAAAA 1.22 0.08
ANP-Oligo 119 AAAAAAAGCUCAAAAAAAAAA 1.26 0.15
ANP-Oligo 035 AAAAAAAGUCAAAAAAAAAAA 1.33 0.28
ANP-Oligo 143 AAAAAAAGGUUCAAAAAAAAAAA 1.35 0.11

Table 4: ssRNA oligonucleotides - Figure 7A

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5' -&gt; 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP-Oligo 194</td>
<td>AAGUCAAAAAAAAAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 195</td>
<td>AAAAAAAGUCAAAAAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 035</td>
<td>AAAAAAAGUCAAAAAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 196</td>
<td>AAAAAAAGUCAAAAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 197</td>
<td>AAAAAAAGUCAAAAAAAAAA</td>
</tr>
</tbody>
</table>

Table 5: ssRNA oligonucleotides - Figure 7B

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5' -&gt; 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP-Oligo 035</td>
<td>AAAAAAAAGUCAAAAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 198</td>
<td>AAAAAAAAGUCAACAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 199</td>
<td>AAAAAAAAGUCAGAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 200</td>
<td>AAAAAAAAGUCAAUAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 083</td>
<td>AAAAAACGUCAAAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 201</td>
<td>AAAAAACGUCACAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 202</td>
<td>AAAAAACGUCAGAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 203</td>
<td>AAAAAACGUCAUAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 131</td>
<td>AAAAAAGGUCAAAARAAA</td>
</tr>
<tr>
<td>ANP-Oligo 204</td>
<td>AAAAAAGGUCACAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 205</td>
<td>AAAAAAGGUCAGAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 206</td>
<td>AAAAAAGGUCAUAAAAAAA</td>
</tr>
<tr>
<td>ANP-Oligo 179</td>
<td>AAAAAAUGUCAAAAAAAAA</td>
</tr>
</tbody>
</table>

ANP-Oligo 133 AAAAAAAGGUUAAAAAAAAA 1,04 0,13
ANP-Oligo 145 AAAAAAAGUUAAAAAAAAAA 1,17 0,08
ANP-Oligo 144 AAAAAAAGGUUGAAAAAAAAA 1,22 0,08
ANP-Oligo 119 AAAAAAAGCUCAAAAAAAAAA 1,26 0,15
ANP-Oligo 035 AAAAAAAGUCAAAAAAAAAAA 1,33 0,28
ANP-Oligo 143 AAAAAAAGGUUCAAAAAAAAAAA 1,35 0,11
<table>
<thead>
<tr>
<th>Oligo-name</th>
<th>Sequence 5' -&gt; 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.2 sense</td>
<td>AGCUUAACCUGCUUCAA</td>
</tr>
<tr>
<td>L7A</td>
<td>AAAAAAACCUGCUUCAA</td>
</tr>
<tr>
<td>L8A</td>
<td>AAAAAAAACUGCUUCAA</td>
</tr>
<tr>
<td>L9A</td>
<td>AAAAAAAAUGCUUCAA</td>
</tr>
<tr>
<td>L10A</td>
<td>AAAAAAAAGCUUCAA</td>
</tr>
<tr>
<td>L11A</td>
<td>AAAAAAAAACUCAUCAA</td>
</tr>
<tr>
<td>L12A</td>
<td>AAAAAAAAACCUUCAA</td>
</tr>
<tr>
<td>R9A</td>
<td>AGCUUAACCUCAAAAAA</td>
</tr>
<tr>
<td>R8A</td>
<td>AGCUUAACCUGAAAAAA</td>
</tr>
<tr>
<td>R7A</td>
<td>AGCUUAACCUGAAAAAA</td>
</tr>
<tr>
<td>R6A</td>
<td>AGCUUAACCUGAAAAAA</td>
</tr>
<tr>
<td>R5A</td>
<td>AGCUUAACCUGAAAAAA</td>
</tr>
<tr>
<td>R4A</td>
<td>AGCUUAACCUGAAAAAA</td>
</tr>
<tr>
<td>R3A</td>
<td>AGCUUAACCUGAAAAAA</td>
</tr>
</tbody>
</table>
**Table 12:** 3mer motifs and their mean levels of IFN-α induction

A. 3mer motifs 5'-NNN-3"

<table>
<thead>
<tr>
<th>Motif</th>
<th>Occurrences</th>
<th>Mean (IFN-α point score)</th>
<th>Sem</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAA</td>
<td>2192</td>
<td>0.00</td>
<td>0.01</td>
<td>0.389</td>
</tr>
<tr>
<td>AAC</td>
<td>67</td>
<td>-0.14</td>
<td>0.02</td>
<td>** 0.001</td>
</tr>
<tr>
<td>AAG</td>
<td>67</td>
<td>0.11</td>
<td>0.06</td>
<td>** 0.009</td>
</tr>
<tr>
<td>AAU</td>
<td>67</td>
<td>0.01</td>
<td>0.03</td>
<td>0.841</td>
</tr>
<tr>
<td>ACA</td>
<td>31</td>
<td>-0.17</td>
<td>0.02</td>
<td>** 0.007</td>
</tr>
<tr>
<td>ACC</td>
<td>19</td>
<td>-0.19</td>
<td>0.01</td>
<td>* 0.017</td>
</tr>
<tr>
<td>ACG</td>
<td>19</td>
<td>-0.12</td>
<td>0.05</td>
<td>0.128</td>
</tr>
<tr>
<td>ACU</td>
<td>19</td>
<td>-0.07</td>
<td>0.05</td>
<td>0.407</td>
</tr>
<tr>
<td>AGA</td>
<td>31</td>
<td>-0.14</td>
<td>0.02</td>
<td>* 0.021</td>
</tr>
<tr>
<td>AGC</td>
<td>19</td>
<td>-0.05</td>
<td>0.09</td>
<td>0.508</td>
</tr>
<tr>
<td>AGG</td>
<td>19</td>
<td>-0.01</td>
<td>0.09</td>
<td>0.877</td>
</tr>
<tr>
<td>AGU</td>
<td>19</td>
<td>0.59</td>
<td>0.10</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>AUA</td>
<td>31</td>
<td>-0.10</td>
<td>0.02</td>
<td>0.118</td>
</tr>
<tr>
<td>AUC</td>
<td>19</td>
<td>-0.11</td>
<td>0.03</td>
<td>0.176</td>
</tr>
<tr>
<td>AUG</td>
<td>19</td>
<td>0.08</td>
<td>0.07</td>
<td>0.318</td>
</tr>
<tr>
<td>AUU</td>
<td>19</td>
<td>0.09</td>
<td>0.06</td>
<td>0.253</td>
</tr>
<tr>
<td>CAA</td>
<td>67</td>
<td>0.00</td>
<td>0.05</td>
<td>0.913</td>
</tr>
<tr>
<td>CAC</td>
<td>7</td>
<td>-0.21</td>
<td>0.01</td>
<td>0.11</td>
</tr>
<tr>
<td>CAG</td>
<td>7</td>
<td>-0.13</td>
<td>0.06</td>
<td>0.313</td>
</tr>
<tr>
<td>CAU</td>
<td>7</td>
<td>-0.19</td>
<td>0.01</td>
<td>0.143</td>
</tr>
<tr>
<td>CCA</td>
<td>19</td>
<td>-0.14</td>
<td>0.05</td>
<td>0.077</td>
</tr>
<tr>
<td>CCC</td>
<td>7</td>
<td>-0.21</td>
<td>0.01</td>
<td>0.102</td>
</tr>
<tr>
<td>CCG</td>
<td>7</td>
<td>-0.21</td>
<td>0.01</td>
<td>0.11</td>
</tr>
<tr>
<td>CCU</td>
<td>7</td>
<td>-0.15</td>
<td>0.03</td>
<td>0.252</td>
</tr>
<tr>
<td>CGA</td>
<td>19</td>
<td>-0.14</td>
<td>0.04</td>
<td>0.082</td>
</tr>
<tr>
<td>CGC</td>
<td>7</td>
<td>-0.19</td>
<td>0.02</td>
<td>0.155</td>
</tr>
<tr>
<td>CGG</td>
<td>7</td>
<td>-0.18</td>
<td>0.03</td>
<td>0.172</td>
</tr>
<tr>
<td>CGU</td>
<td>7</td>
<td>0.05</td>
<td>0.11</td>
<td>0.678</td>
</tr>
<tr>
<td>CUA</td>
<td>19</td>
<td>-0.09</td>
<td>0.05</td>
<td>0.251</td>
</tr>
<tr>
<td>-----</td>
<td>-----</td>
<td>-------</td>
<td>------</td>
<td>-------</td>
</tr>
<tr>
<td>CUC</td>
<td>7</td>
<td>0.16</td>
<td>0.19</td>
<td>0.229</td>
</tr>
<tr>
<td>CUG</td>
<td>7</td>
<td>0.00</td>
<td>0.12</td>
<td>0.979</td>
</tr>
<tr>
<td>CUU</td>
<td>7</td>
<td>0.08</td>
<td>0.12</td>
<td>0.529</td>
</tr>
<tr>
<td>GAA</td>
<td>67</td>
<td>-0.08</td>
<td>0.03</td>
<td>* 0.044</td>
</tr>
<tr>
<td>GAC</td>
<td>7</td>
<td>-0.19</td>
<td>0.02</td>
<td>0.153</td>
</tr>
<tr>
<td>GAG</td>
<td>7</td>
<td>-0.13</td>
<td>0.06</td>
<td>0.306</td>
</tr>
<tr>
<td>GAU</td>
<td>7</td>
<td>-0.11</td>
<td>0.01</td>
<td>0.385</td>
</tr>
<tr>
<td>GCA</td>
<td>19</td>
<td>-0.12</td>
<td>0.04</td>
<td>0.114</td>
</tr>
<tr>
<td>GCC</td>
<td>7</td>
<td>-0.19</td>
<td>0.03</td>
<td>0.149</td>
</tr>
<tr>
<td>GCG</td>
<td>7</td>
<td>-0.17</td>
<td>0.03</td>
<td>0.184</td>
</tr>
<tr>
<td>GCU</td>
<td>7</td>
<td>0.22</td>
<td>0.21</td>
<td>0.094</td>
</tr>
<tr>
<td>GGA</td>
<td>19</td>
<td>-0.13</td>
<td>0.04</td>
<td>0.111</td>
</tr>
<tr>
<td>GGC</td>
<td>7</td>
<td>-0.17</td>
<td>0.04</td>
<td>0.194</td>
</tr>
<tr>
<td>GGG</td>
<td>7</td>
<td>-0.22</td>
<td>0.00</td>
<td>0.095</td>
</tr>
<tr>
<td>GGU</td>
<td>7</td>
<td>0.40</td>
<td>0.18</td>
<td>** 0.002</td>
</tr>
<tr>
<td>GUA</td>
<td>19</td>
<td>0.27</td>
<td>0.08</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>GUC</td>
<td>7</td>
<td>0.82</td>
<td>0.10</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>GUG</td>
<td>7</td>
<td>0.32</td>
<td>0.13</td>
<td>* 0.014</td>
</tr>
<tr>
<td>GUU</td>
<td>7</td>
<td>0.87</td>
<td>0.18</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>UAA</td>
<td>67</td>
<td>0.06</td>
<td>0.04</td>
<td>0.135</td>
</tr>
<tr>
<td>UAC</td>
<td>7</td>
<td>-0.06</td>
<td>0.08</td>
<td>0.647</td>
</tr>
<tr>
<td>UAG</td>
<td>7</td>
<td>0.01</td>
<td>0.08</td>
<td>0.94</td>
</tr>
<tr>
<td>UAU</td>
<td>7</td>
<td>-0.03</td>
<td>0.07</td>
<td>0.793</td>
</tr>
<tr>
<td>UCA</td>
<td>19</td>
<td>0.35</td>
<td>0.12</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>UCC</td>
<td>7</td>
<td>-0.03</td>
<td>0.14</td>
<td>0.794</td>
</tr>
<tr>
<td>UCG</td>
<td>7</td>
<td>0.02</td>
<td>0.09</td>
<td>0.848</td>
</tr>
<tr>
<td>UCU</td>
<td>7</td>
<td>0.10</td>
<td>0.13</td>
<td>0.421</td>
</tr>
<tr>
<td>UGA</td>
<td>19</td>
<td>0.04</td>
<td>0.08</td>
<td>0.611</td>
</tr>
<tr>
<td>UGC</td>
<td>7</td>
<td>0.01</td>
<td>0.07</td>
<td>0.91</td>
</tr>
<tr>
<td>UGG</td>
<td>7</td>
<td>0.10</td>
<td>0.09</td>
<td>0.448</td>
</tr>
<tr>
<td>UGU</td>
<td>7</td>
<td>0.69</td>
<td>0.06</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>UUA</td>
<td>19</td>
<td>0.17</td>
<td>0.10</td>
<td>* 0.036</td>
</tr>
<tr>
<td>UUC</td>
<td>7</td>
<td>0.37</td>
<td>0.18</td>
<td>** 0.005</td>
</tr>
</tbody>
</table>
B. 3mer motifs 5'-NN-N-3"

<table>
<thead>
<tr>
<th>Motif</th>
<th>Occurrences</th>
<th>Mean</th>
<th>Sem</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AANA</td>
<td>1864</td>
<td>-0.01</td>
<td>0.01</td>
<td>0.096</td>
</tr>
<tr>
<td>AANC</td>
<td>112</td>
<td>-0.12</td>
<td>0.02</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>AANG</td>
<td>112</td>
<td>0.07</td>
<td>0.04</td>
<td>* 0.021</td>
</tr>
<tr>
<td>AANU</td>
<td>112</td>
<td>0.11</td>
<td>0.03</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>ACNA</td>
<td>40</td>
<td>-0.17</td>
<td>0.02</td>
<td>** 0.002</td>
</tr>
<tr>
<td>ACNC</td>
<td>16</td>
<td>-0.18</td>
<td>0.02</td>
<td>* 0.035</td>
</tr>
<tr>
<td>ACNG</td>
<td>16</td>
<td>-0.10</td>
<td>0.06</td>
<td>0.244</td>
</tr>
<tr>
<td>ACNU</td>
<td>16</td>
<td>-0.05</td>
<td>0.06</td>
<td>0.533</td>
</tr>
<tr>
<td>AGNA</td>
<td>40</td>
<td>-0.09</td>
<td>0.03</td>
<td>0.111</td>
</tr>
<tr>
<td>AGNC</td>
<td>16</td>
<td>0.06</td>
<td>0.12</td>
<td>0.46</td>
</tr>
<tr>
<td>AGNG</td>
<td>16</td>
<td>-0.02</td>
<td>0.09</td>
<td>0.805</td>
</tr>
<tr>
<td>AGNU</td>
<td>16</td>
<td>0.52</td>
<td>0.15</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>AUNA</td>
<td>40</td>
<td>-0.11</td>
<td>0.02</td>
<td>* 0.038</td>
</tr>
<tr>
<td>AUNC</td>
<td>16</td>
<td>-0.07</td>
<td>0.04</td>
<td>0.398</td>
</tr>
<tr>
<td>AUNG</td>
<td>16</td>
<td>0.05</td>
<td>0.07</td>
<td>0.556</td>
</tr>
<tr>
<td>AUNU</td>
<td>16</td>
<td>0.19</td>
<td>0.08</td>
<td>* 0.027</td>
</tr>
<tr>
<td>CANA</td>
<td>76</td>
<td>-0.02</td>
<td>0.04</td>
<td>0.668</td>
</tr>
<tr>
<td>CANC</td>
<td>4</td>
<td>-0.22</td>
<td>0.01</td>
<td>0.209</td>
</tr>
<tr>
<td>CANG</td>
<td>4</td>
<td>-0.22</td>
<td>0.00</td>
<td>0.201</td>
</tr>
<tr>
<td>CANU</td>
<td>4</td>
<td>-0.10</td>
<td>0.11</td>
<td>0.577</td>
</tr>
<tr>
<td>CCNA</td>
<td>28</td>
<td>-0.16</td>
<td>0.04</td>
<td>* 0.015</td>
</tr>
<tr>
<td>CCNC</td>
<td>4</td>
<td>-0.16</td>
<td>0.05</td>
<td>0.365</td>
</tr>
<tr>
<td>CCNG</td>
<td>4</td>
<td>-0.21</td>
<td>0.01</td>
<td>0.216</td>
</tr>
<tr>
<td>CCNU</td>
<td>4</td>
<td>-0.18</td>
<td>0.02</td>
<td>0.295</td>
</tr>
<tr>
<td>CGNA</td>
<td>28</td>
<td>-0.14</td>
<td>0.03</td>
<td>* 0.036</td>
</tr>
<tr>
<td>CGNC</td>
<td>4</td>
<td>0.00</td>
<td>0.23</td>
<td>0.981</td>
</tr>
<tr>
<td>CGNG</td>
<td>4</td>
<td>-0.19</td>
<td>0.03</td>
<td>0.268</td>
</tr>
<tr>
<td>CGNU</td>
<td>4</td>
<td>-0.05</td>
<td>0.03</td>
<td>0.765</td>
</tr>
<tr>
<td>CUNA</td>
<td>28</td>
<td>0.01</td>
<td>0.07</td>
<td>0.896</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>CUNC</td>
<td>4</td>
<td>-0.08</td>
<td>0.10</td>
<td>0.649</td>
</tr>
<tr>
<td>CUNG</td>
<td>4</td>
<td>-0.09</td>
<td>0.04</td>
<td>0.616</td>
</tr>
<tr>
<td>CUNU</td>
<td>4</td>
<td>0.10</td>
<td>0.21</td>
<td>0.567</td>
</tr>
<tr>
<td>GANA</td>
<td>76</td>
<td>-0.09</td>
<td>0.03</td>
<td>*0.017</td>
</tr>
<tr>
<td>GANC</td>
<td>4</td>
<td>-0.17</td>
<td>0.03</td>
<td>0.324</td>
</tr>
<tr>
<td>GANG</td>
<td>4</td>
<td>-0.19</td>
<td>0.02</td>
<td>0.281</td>
</tr>
<tr>
<td>GANU</td>
<td>4</td>
<td>-0.03</td>
<td>0.08</td>
<td>0.87</td>
</tr>
<tr>
<td>GCNA</td>
<td>28</td>
<td>-0.13</td>
<td>0.03</td>
<td>*0.044</td>
</tr>
<tr>
<td>GCNC</td>
<td>4</td>
<td>0.15</td>
<td>0.37</td>
<td>0.38</td>
</tr>
<tr>
<td>GCNG</td>
<td>4</td>
<td>-0.18</td>
<td>0.04</td>
<td>0.298</td>
</tr>
<tr>
<td>GCNU</td>
<td>4</td>
<td>0.10</td>
<td>0.22</td>
<td>0.548</td>
</tr>
<tr>
<td>GGNA</td>
<td>28</td>
<td>-0.10</td>
<td>0.04</td>
<td>0.131</td>
</tr>
<tr>
<td>GGNC</td>
<td>4</td>
<td>0.07</td>
<td>0.29</td>
<td>0.697</td>
</tr>
<tr>
<td>GGNG</td>
<td>4</td>
<td>-0.12</td>
<td>0.10</td>
<td>0.492</td>
</tr>
<tr>
<td>GGNU</td>
<td>4</td>
<td>0.16</td>
<td>0.30</td>
<td>0.353</td>
</tr>
<tr>
<td>GUNA</td>
<td>28</td>
<td>0.38</td>
<td>0.08</td>
<td>**&lt;0.001</td>
</tr>
<tr>
<td>GUNC</td>
<td>4</td>
<td>0.72</td>
<td>0.23</td>
<td>**&lt;0.001</td>
</tr>
<tr>
<td>GUNG</td>
<td>4</td>
<td>0.59</td>
<td>0.21</td>
<td>**&lt;0.001</td>
</tr>
<tr>
<td>GUNU</td>
<td>4</td>
<td>0.83</td>
<td>0.17</td>
<td>**&lt;0.001</td>
</tr>
<tr>
<td>UANA</td>
<td>76</td>
<td>0.06</td>
<td>0.04</td>
<td>0.15</td>
</tr>
<tr>
<td>UANC</td>
<td>4</td>
<td>-0.09</td>
<td>0.02</td>
<td>0.599</td>
</tr>
<tr>
<td>UANG</td>
<td>4</td>
<td>-0.08</td>
<td>0.02</td>
<td>0.641</td>
</tr>
<tr>
<td>UANU</td>
<td>4</td>
<td>0.00</td>
<td>0.11</td>
<td>0.999</td>
</tr>
<tr>
<td>UCNA</td>
<td>28</td>
<td>0.30</td>
<td>0.10</td>
<td>**&lt;0.001</td>
</tr>
<tr>
<td>UCNC</td>
<td>4</td>
<td>-0.06</td>
<td>0.10</td>
<td>0.738</td>
</tr>
<tr>
<td>UCNG</td>
<td>4</td>
<td>-0.11</td>
<td>0.04</td>
<td>0.541</td>
</tr>
<tr>
<td>UCNU</td>
<td>4</td>
<td>-0.07</td>
<td>0.05</td>
<td>0.706</td>
</tr>
<tr>
<td>UGNA</td>
<td>28</td>
<td>0.17</td>
<td>0.07</td>
<td>**0.008</td>
</tr>
<tr>
<td>UGNC</td>
<td>4</td>
<td>0.16</td>
<td>0.19</td>
<td>0.351</td>
</tr>
<tr>
<td>UGNG</td>
<td>4</td>
<td>0.04</td>
<td>0.14</td>
<td>0.817</td>
</tr>
<tr>
<td>UGNU</td>
<td>4</td>
<td>0.20</td>
<td>0.19</td>
<td>0.239</td>
</tr>
<tr>
<td>UUANA</td>
<td>28</td>
<td>0.30</td>
<td>0.09</td>
<td>**&lt;0.001</td>
</tr>
<tr>
<td>UUNC</td>
<td>4</td>
<td>0.15</td>
<td>0.14</td>
<td>0.377</td>
</tr>
<tr>
<td>UUNG</td>
<td>4</td>
<td>0.19</td>
<td>0.11</td>
<td>0.267</td>
</tr>
<tr>
<td>UUNU</td>
<td>4</td>
<td>0.21</td>
<td>0.18</td>
<td>0.228</td>
</tr>
</tbody>
</table>
C. 3mer motifs 5'-N-NN-3'

<table>
<thead>
<tr>
<th>Motif</th>
<th>Occurences</th>
<th>Mean</th>
<th>Sem</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANAA</td>
<td>1864</td>
<td>-0.01</td>
<td>0.01</td>
<td>0.107</td>
</tr>
<tr>
<td>ANAC</td>
<td>76</td>
<td>-0.14</td>
<td>0.02</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>ANAG</td>
<td>76</td>
<td>0.09</td>
<td>0.05</td>
<td>* 0.019</td>
</tr>
<tr>
<td>ANAU</td>
<td>76</td>
<td>-0.01</td>
<td>0.03</td>
<td>0.78</td>
</tr>
<tr>
<td>ANCA</td>
<td>40</td>
<td>-0.18</td>
<td>0.01</td>
<td>** 0.001</td>
</tr>
<tr>
<td>ANCC</td>
<td>28</td>
<td>-0.20</td>
<td>0.01</td>
<td>** 0.003</td>
</tr>
<tr>
<td>ANCG</td>
<td>28</td>
<td>-0.13</td>
<td>0.03</td>
<td>* 0.048</td>
</tr>
<tr>
<td>ANCU</td>
<td>28</td>
<td>0.02</td>
<td>0.07</td>
<td>0.767</td>
</tr>
<tr>
<td>ANGA</td>
<td>40</td>
<td>-0.16</td>
<td>0.01</td>
<td>** 0.004</td>
</tr>
<tr>
<td>ANGC</td>
<td>28</td>
<td>-0.08</td>
<td>0.06</td>
<td>0.207</td>
</tr>
<tr>
<td>ANGG</td>
<td>28</td>
<td>-0.04</td>
<td>0.07</td>
<td>0.533</td>
</tr>
<tr>
<td>ANGU</td>
<td>28</td>
<td>0.57</td>
<td>0.08</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>ANUA</td>
<td>40</td>
<td>-0.05</td>
<td>0.03</td>
<td>0.366</td>
</tr>
<tr>
<td>ANUC</td>
<td>28</td>
<td>0.06</td>
<td>0.07</td>
<td>0.334</td>
</tr>
<tr>
<td>ANUG</td>
<td>28</td>
<td>0.18</td>
<td>0.07</td>
<td>** 0.007</td>
</tr>
<tr>
<td>ANUU</td>
<td>28</td>
<td>0.27</td>
<td>0.08</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>CNAA</td>
<td>112</td>
<td>-0.04</td>
<td>0.03</td>
<td>0.218</td>
</tr>
<tr>
<td>CNAC</td>
<td>4</td>
<td>-0.21</td>
<td>0.01</td>
<td>0.215</td>
</tr>
<tr>
<td>CNAG</td>
<td>4</td>
<td>-0.19</td>
<td>0.03</td>
<td>0.265</td>
</tr>
<tr>
<td>CNAU</td>
<td>4</td>
<td>-0.16</td>
<td>0.03</td>
<td>0.361</td>
</tr>
<tr>
<td>CNCA</td>
<td>16</td>
<td>-0.05</td>
<td>0.09</td>
<td>0.537</td>
</tr>
<tr>
<td>CNCC</td>
<td>4</td>
<td>-0.21</td>
<td>0.01</td>
<td>0.218</td>
</tr>
<tr>
<td>CNCG</td>
<td>4</td>
<td>-0.20</td>
<td>0.02</td>
<td>0.258</td>
</tr>
<tr>
<td>CNCU</td>
<td>4</td>
<td>-0.17</td>
<td>0.02</td>
<td>0.334</td>
</tr>
<tr>
<td>CNGA</td>
<td>16</td>
<td>-0.18</td>
<td>0.02</td>
<td>* 0.039</td>
</tr>
<tr>
<td>CNGC</td>
<td>4</td>
<td>-0.19</td>
<td>0.02</td>
<td>0.262</td>
</tr>
<tr>
<td>CNGG</td>
<td>4</td>
<td>-0.20</td>
<td>0.02</td>
<td>0.251</td>
</tr>
<tr>
<td>CNGU</td>
<td>4</td>
<td>0.20</td>
<td>0.19</td>
<td>0.244</td>
</tr>
<tr>
<td>CNUA</td>
<td>16</td>
<td>-0.07</td>
<td>0.06</td>
<td>0.389</td>
</tr>
<tr>
<td>CNUC</td>
<td>4</td>
<td>0.17</td>
<td>0.19</td>
<td>0.318</td>
</tr>
<tr>
<td>CNUG</td>
<td>4</td>
<td>-0.13</td>
<td>0.06</td>
<td>0.464</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>CNUU</td>
<td>4</td>
<td>-0.11</td>
<td>0.05</td>
<td>0.538</td>
</tr>
<tr>
<td>GNAA</td>
<td>112</td>
<td>-0.04</td>
<td>0.03</td>
<td>0.186</td>
</tr>
<tr>
<td>GNAC</td>
<td>4</td>
<td>-0.06</td>
<td>0.14</td>
<td>0.717</td>
</tr>
<tr>
<td>GNAG</td>
<td>4</td>
<td>-0.08</td>
<td>0.12</td>
<td>0.649</td>
</tr>
<tr>
<td>GNAU</td>
<td>4</td>
<td>0.02</td>
<td>0.11</td>
<td>0.89</td>
</tr>
<tr>
<td>GNCA</td>
<td>16</td>
<td>0.10</td>
<td>0.13</td>
<td>0.243</td>
</tr>
<tr>
<td>GNCC</td>
<td>4</td>
<td>0.03</td>
<td>0.25</td>
<td>0.842</td>
</tr>
<tr>
<td>GNCG</td>
<td>4</td>
<td>-0.06</td>
<td>0.17</td>
<td>0.745</td>
</tr>
<tr>
<td>GNCU</td>
<td>4</td>
<td>0.11</td>
<td>0.24</td>
<td>0.534</td>
</tr>
<tr>
<td>GNGA</td>
<td>16</td>
<td>-0.11</td>
<td>0.05</td>
<td>0.203</td>
</tr>
<tr>
<td>GNGC</td>
<td>4</td>
<td>-0.07</td>
<td>0.15</td>
<td>0.688</td>
</tr>
<tr>
<td>GNGG</td>
<td>4</td>
<td>-0.07</td>
<td>0.15</td>
<td>0.706</td>
</tr>
<tr>
<td>GNGU</td>
<td>4</td>
<td>0.22</td>
<td>0.26</td>
<td>0.207</td>
</tr>
<tr>
<td>GNUA</td>
<td>16</td>
<td>0.13</td>
<td>0.10</td>
<td>0.14</td>
</tr>
<tr>
<td>GNUC</td>
<td>4</td>
<td>0.87</td>
<td>0.33</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>GNUG</td>
<td>4</td>
<td>0.30</td>
<td>0.31</td>
<td>0.08</td>
</tr>
<tr>
<td>GNUU</td>
<td>4</td>
<td>0.72</td>
<td>0.29</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>UNAA</td>
<td>112</td>
<td>0.14</td>
<td>0.04</td>
<td>** &lt; 0.001</td>
</tr>
<tr>
<td>UNAC</td>
<td>4</td>
<td>-0.09</td>
<td>0.04</td>
<td>0.603</td>
</tr>
<tr>
<td>UNAG</td>
<td>4</td>
<td>-0.08</td>
<td>0.01</td>
<td>0.626</td>
</tr>
<tr>
<td>UNAU</td>
<td>4</td>
<td>-0.11</td>
<td>0.02</td>
<td>0.522</td>
</tr>
<tr>
<td>UNCA</td>
<td>16</td>
<td>0.18</td>
<td>0.11</td>
<td>* 0.032</td>
</tr>
<tr>
<td>UNCC</td>
<td>4</td>
<td>-0.11</td>
<td>0.03</td>
<td>0.528</td>
</tr>
<tr>
<td>UNCG</td>
<td>4</td>
<td>-0.04</td>
<td>0.09</td>
<td>0.807</td>
</tr>
<tr>
<td>UNCU</td>
<td>4</td>
<td>-0.08</td>
<td>0.05</td>
<td>0.634</td>
</tr>
<tr>
<td>UNGA</td>
<td>16</td>
<td>0.14</td>
<td>0.09</td>
<td>0.113</td>
</tr>
<tr>
<td>UNGC</td>
<td>4</td>
<td>-0.01</td>
<td>0.06</td>
<td>0.975</td>
</tr>
<tr>
<td>UNGG</td>
<td>4</td>
<td>-0.03</td>
<td>0.09</td>
<td>0.86</td>
</tr>
<tr>
<td>UNGU</td>
<td>4</td>
<td>0.39</td>
<td>0.14</td>
<td>* 0.025</td>
</tr>
<tr>
<td>UNUA</td>
<td>16</td>
<td>0.29</td>
<td>0.12</td>
<td>** 0.001</td>
</tr>
<tr>
<td>UNUC</td>
<td>4</td>
<td>0.37</td>
<td>0.17</td>
<td>* 0.032</td>
</tr>
<tr>
<td>UNUG</td>
<td>4</td>
<td>0.20</td>
<td>0.14</td>
<td>0.24</td>
</tr>
<tr>
<td>UNUU</td>
<td>4</td>
<td>0.15</td>
<td>0.17</td>
<td>0.375</td>
</tr>
</tbody>
</table>
**Table 13:** RNA oligonucleotide containing multiple copies of GUCA

<table>
<thead>
<tr>
<th>Oligo-name</th>
<th>Sequence 5' → 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.2 sense</td>
<td>AGCUUAACCUGUCCUUCA</td>
</tr>
<tr>
<td>Poly GUCA</td>
<td>GUCAAGUCAAGUCAAGUCA</td>
</tr>
<tr>
<td>ANP35</td>
<td>AAAAAAAAAAGUCAAAAAAAA</td>
</tr>
</tbody>
</table>
Claims 1-43

1. A method for determining the immunostimulatory activity of a RNA oligonucleotide, comprising the steps of:
   (a) complexing the RNA oligonucleotide with a complexation agent;
   (b) contacting a cell with the complexed RNA oligonucleotide, wherein the cell expresses TLR7, TLR8, or both TLR7 and TLR8; and
   (c) determining the amount of IFN-α produced by the cell of step (b), an increase of IFN-α production indicating immunostimulatory activity of the RNA oligonucleotide.

2. The method of claim 1, wherein the complexation agent is a polycationic peptide, preferably poly-L-arginine (poly-L-Arg).

3. The method of claim 1, wherein the cell is a peripheral blood mononuclear cell (PBMC), preferably a plasmacytoid dendritic cell (PDC).

4. A method for predicting the immunostimulatory activity of a RNA oligonucleotide, comprising the steps of:
   (a) identifying all possible 3-nucleotide (3mer) motifs contained in the oligonucleotide;
   (b) assigning an IFN-α point score for each individual 3mer motif:
      (i) for a 3mer motif which appears in Table 7, assign an IFN-α point score according to Table 7;
      (ii) for a 3mer motif which does not appear in Table 7, assign an IFN-α point score of 0;
   (c) assigning the sum of the IFN-α point scores of individual 3mer motifs as the IFN-α score of the oligonucleotide; and
   (d) assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least 23, an intermediate immunostimulatory activity if the IFN-α score is between -4 and 23, and a low immunostimulatory activity if the IFN-α score is at most -4, when n=6;
assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least 26, an intermediate immunostimulatory activity if the IFN-α score is
between -4 and 26, and a low immunostimulatory activity if the IFN-α score is at most -4, when n=7;

assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least 28, an intermediate immunostimulatory activity if the IFN-α score is between -5 and 28, and a low immunostimulatory activity if the IFN-α score is at most -5, when n=8;

assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least 30, an intermediate immunostimulatory activity if the IFN-α score is between -5 and 30, and a low immunostimulatory activity if the IFN-α score is at most -5, when n=9;

assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least $1.4909 \times n + 22.014$, an intermediate immunostimulatory activity if the IFN-α score is between $0.005 \times n^2 - 0.2671 \times n - 3.5531$ and $1.4909 \times n + 22.014$, and a low immunostimulatory activity if the IFN-α score is at most $0.005 \times n^2 - 0.2671 \times n - 3.5531$, when n is greater than 9,

wherein n is the length of the oligonucleotide.

5. A method for predicting the immunostimulatory activity of a RNA oligonucleotide, comprising the steps of:
(a) identifying all possible 3-nucleotide (3mer) motifs contained in the oligonucleotide;
(b) assigning an IFN-α point score for each individual 3mer motif according to Table 12A;
(c) assigning the highest individual IFN-α point score as the IFN-α score of the oligonucleotide; and
(d) assigning to the oligonucleotide a high immunostimulatory activity if the IFN-α score is at least 0.58, an intermediate immunostimulatory activity if the IFN-α score is between 0.11 and 0.58, and a low immunostimulatory activity if the IFN-α score is at most 0.11.

6. A method for preparing a RNA oligonucleotide having high immunostimulatory activity, comprising the steps of:
(a) providing candidate oligonucleotide sequence(s);
(b) identifying oligonucleotide sequence(s) with high immunostimulatory activity predicted according to the method of claim 4 or 5;
(c) preparing the RNA oligonucleotide(s) identified for high immunostimulatory activity in step (b); and
(d) optionally testing the immunostimulatory activity of the RNA oligonucleotide(s) prepared in step (c) according to the method of claim 1; and
(e) further optionally modifying the oligonucleotide(s) to optimize the immunostimulatory activity.

7. A method for preparing a RNA oligonucleotide having low immunostimulatory activity, comprising the steps of:
(a) providing candidate oligonucleotide sequence(s);
(b) identifying oligonucleotide sequence(s) with low immunostimulatory activity predicted according to the method of claim 4 or 5;
(c) preparing the RNA oligonucleotide(s) identified for low immunostimulatory activity in step (b); and
(d) optionally testing the RNA oligonucleotide(s) prepared in step (c) for the lack of immunostimulatory activity according to the method of claim 1; and
(e) further optionally modifying the oligonucleotide(s) to minimize the immunostimulatory activity.

8. A method for preparing a RNA oligonucleotide having high immunostimulatory activity, comprising the steps of:
(a) providing an oligonucleotide sequence which comprises at least one, preferably at least two, more preferably at least three, even more preferably at least four, of the 4-nucleotide (4mer) motifs selected from the group consisting of:

- GUUC (No. 1),
- GUCA (No. 2),
- GCUC (No. 3),
- GUUG (No. 4),
- GUUU (No. 5),
- GGGU (No. 6),
- GUGU (No. 7),
- GGUC (No. 8),
- GUCU (No. 9),
- GUCC (No. 10),
- GCUU (No. 11),
- UUGU (No. 12),
- UGUC (No. 13),
- CGU (No. 14),
- CGUC (No. 15),
- UGGU (No. 16),
- GUA (No. 17),
- UGUA (No. 18),
- UUUC (No. 19),
- UGGG (No. 20),
- GUGA (No. 21),
- GUCG (No. 22),
- UUGG (No. 23),
- UGGU (No. 24),
- GUGG (No. 25),
- GUCG (No. 26),
- GUAC (No. 27),
- GUAU (No. 28),
- UAGU (No. 29),
- GUAG (No. 30),
- UCCA (No. 31),
- UUGG (No. 32),
- UCUC (No. 33),
- CAGU (No. 34),
- UUGC (No. 35),
- CUUC (No. 36),
- GAGU (No. 37),
- GGUG (No. 38),
- UUGC (No. 39),
- UUUU (No. 40),
- CUCA (No. 41),
- UCGU (No. 42),
- UUCU (No. 43),
- UGCG (No. 44),
- CGU (No. 45),
- CUUG (No. 46),
- UUAC (No. 47),
wherein the nucleotide sequences of the motifs are 5' -> 3',

wherein the oligonucleotide is between 6 and 64, preferably between 12 and 50, more preferably between 14 and 40, even more preferably between 16 and 36, and most preferably between 18 and 25 nucleotides in length,

wherein at least one strand of the RNA oligonucleotide has an IFN-α score of at least 23 when n=6; at least 26 when n=7; at least 28 when n=8; at least 30 when n=9; at least 1.4909 x n + 22.014 when n is greater than 9, wherein the IFN-α score is assigned according to the method as defined in Claim 4(a)-(c), wherein n is the length of the oligonucleotide,

or wherein at least one strand of the RNA oligonucleotide has an IFN-α score of at least 0.58, wherein the IFN-α score is assigned according to the method as defined in Claim 5(a)-(c),

(b) preparing the RNA oligonucleotide of step (a); and

(c) optionally testing the immunostimulatory activity of the RNA oligonucleotide prepared in step (b) according to the method of claim 1; and

d) further optionally modifying the oligonucleotide to optimize the immunostimulatory activity.

9. A RNA oligonucleotide having immunostimulatory activity comprising at least one, preferably at least two, more preferably at least three, even more preferably at least four, of the 4-nucleotide (4mer) motifs selected from the group consisting of:

GUUC (No. 1), GUCA (No. 2), GCUC (No. 3), GUUG (No. 4), GUUU (No. 5),
GGUU (No. 6), GUGU (No. 7), GGUC (No. 8), GUCU (No. 9), GUCC (No. 10),
GCUU (No. 11), UUGU (No. 12), UGUC (No. 13), CUGU (No. 14), CGUC (No. 15),
UGUU (No. 16), GUUA (No. 17), UGUU (No. 18), UUUC (No. 19), UUGU (No. 20),
GGUA (No. 21), GUGC (No. 22), UUUG (No. 23), UGGU (No. 24), GUGG (No. 25),
GUGC (No. 26), GUAC (No. 27), GUAU (No. 28), UAGU (No. 29), GUAG (No. 30),
UUCA (No. 31), UUGG (No. 32), UCUC (No. 33), CAGU (No. 34), UUCG (No. 35),
CUUC (No. 36), GAGU (No. 37), GGUG (No. 38), UUGC (No. 39), UUUU (No. 40),
CUCA (No. 41), UCGU (No. 42), UUCU (No. 43), UGCG (No. 44), CGUU (No. 45),
CUUG (No. 46), UUAC (No. 47),

wherein the nucleotide sequences of the motifs are 5' -> 3',

wherein the oligonucleotide is between 6 and 64, preferably between 12 and 50, more preferably between 14 and 40, even more preferably between 16 and 36, and most preferably between 18 and 25 nucleotides in length,
wherein at least one strand of the RNA oligonucleotide has an IFN-α score of at least 23 when n=6; at least 26 when n=7; at least 28 when n=8; at least 30 when n=9; at least 1.4909 x n + 22.014 when n is greater than 9, wherein the IFN-α score is assigned according to the method as defined in Claim 4(a)-(c), wherein n is the length of the oligonucleotide,

or wherein at least one strand of the RNA oligonucleotide has an IFN-α score of at least 0.58, wherein the IFN-α score is assigned according to the method as defined in Claim 5(a)-(c),

and wherein the oligonucleotide is not 5’-UUGAUGUGUUAGUCGU-3’, 5’-GCACCACUAGUGUGUCGCUA-3’, 5’-GUUGUAGUUGUACUCCAGC-3’, 5’-GCCCGUCUGUUGUUGUGACUC-3’, 5’-GUUGUGGUUGUGGUUGUG-3’.

10. The oligonucleotide of claim 9, wherein the 4mer motifs are selected from the group consisting of number 1-19, preferably, number 1-6 of the 4mer motifs.

11. A RNA oligonucleotide having immunostimulatory activity comprising at least one, preferably at least two, more preferably at least three, even more preferably at least four, of the 4mer motifs selected from the group consisting of number 1-6 of the 4mer motifs, wherein the spacer nucleotides which are not part of any of the 4mer motif(s) are identical, and wherein the spacer nucleotide is selected from the group consisting of A, T, C, G and variants and derivatives thereof.

12. The oligonucleotide of any one of claims 9-11, wherein the oligonucleotide does not have gene silencing activity for any known mammalian gene.

13. The oligonucleotide of any one of claims 9-12, wherein the oligonucleotide is single-stranded.

14. The oligonucleotide of claim 13, wherein the oligonucleotide contains a self-complementary sequence and can form a hairpin structure.

15. The oligonucleotide of any one of claims 9-12, wherein the oligonucleotide is double-stranded.
16. The oligonucleotide of any one of claims 9-12, wherein the oligonucleotide is partially double-stranded.

17. The oligonucleotide of any of claims 9-12, wherein the oligonucleotide comprises a phosphorothioate linkage.

18. The oligonucleotide of any of claims 9-12, wherein the oligonucleotide comprises a pyrophosphate linkage.

19. The oligonucleotide of any one of claims 9-18, wherein the oligonucleotide is covalently linked to one or more lipophilic groups.

20. The oligonucleotide of claim 19, wherein the lipophilic group is cholesterol or a derivative thereof.

21. A RNA oligonucleotide having both gene silencing activity and high immunostimulatory activity, wherein at least one strand of the oligonucleotide has an IFN-α score of at least $1.4909 \times n + 31.014$, wherein the IFN-α score is assigned according to the method as defined in Claim 4(a)-(c), wherein $n$ is the length of the oligonucleotide, or wherein at least one strand of the oligonucleotide has an IFN-α score of at least 0.58, wherein the IFN-α score is assigned according to the method as defined in Claim 5(a)-(c), wherein the oligonucleotide is an siRNA and $n$ is between 14 and 25, or wherein the oligonucleotide is an antisense and $n$ is between 14 and 50.

22. A RNA oligonucleotide having gene silencing activity and low immunostimulatory activity, wherein all strand(s) of the oligonucleotide has(have) an IFN-α score of at most $0.6075 \times -9.9484$, wherein the IFN-α score is assigned according to the method as defined in Claim 4(a)-(c), wherein $n$ is the length of the oligonucleotide, or wherein all strand(s) of the oligonucleotide has(have) an IFN-α score of at most 0.11, wherein the IFN-α score is assigned according to the method as defined in Claim 5(a)-(c), wherein the oligonucleotide is an siRNA and $n$ is between 19 and 25.
or wherein the oligonucleotide is an antisense and \( n \) is between 18 and 50.

23. A pharmaceutical composition comprising one or more of the RNA oligonucleotides of any one of claims 9-22 and a pharmaceutically acceptable carrier.

24. The pharmaceutical composition of claim 23, further comprising a RNA complexation agent.

25. The pharmaceutical composition of claim 24, wherein the RNA complexation agent is a polycationic peptide, preferably poly-L-Arg.

26. The pharmaceutical composition of any one of claims 23-25, further comprising one or more further pharmaceutically active agents, wherein the further pharmaceutically active agent is selected from the group consisting of agents that are used to treat cancer, dermatological disorders, immune disorders, metabolic disorders, neurological disorders, ocular diseases, infections, and other hereditary and non-hereditary disorders in a mammal.

27. The pharmaceutical composition of any one of claims 23-25, wherein the composition is for administration selected from the group consisting of airway, oral, ocular, parenteral (including intravenous, intradermal, intramuscular, intraperitoneal, and subcutaneous), rectal, vaginal and topical (including buccal and sublingual) administration.

28. Use of the RNA oligonucleotide of any one of claims 9-20 for the preparation of a pharmaceutical composition for inducing an immune response in a mammal.

29. Use of the RNA oligonucleotide of any one of claims 9-20 for the preparation of a pharmaceutical composition for preventing and/or treating a disorder selected from the group consisting of immune disorders, infections, and cancers in a mammal.

30. Use of the RNA oligonucleotide of claim 21 for the preparation of a pharmaceutical composition for preventing and/or treating a disorder selected from the group consisting of infections and cancers in a mammal.
31. Use of the RNA oligonucleotide of claim 22 for the preparation of a pharmaceutical composition for preventing and/or treating a disorder selected from the group consisting of cancer, dermatological disorders, immune disorders, metabolic disorders, neurological disorders, ocular diseases, infections, and other hereditary and non-hereditary disorders.

32. The use of claim 29 or 31, wherein the immune disorder is selected from the group consisting of allergy, autoimmune disorders, inflammatory disorders, and immunodeficiency.

33. The use of any one of claims 29-31, wherein the infection is selected from the group consisting of viral infections, bacterial infections, anthrax, parasitic infections, fungal infections and prion infection.

34. The use of claim 33, wherein the viral infection is selected from the group consisting of chornic hepatitis B, chornic hepatitis C, HIV infection, RSV infection, HSV infection, VSV infection, CMV infection and influenza infection.


36. The use of any one of claims 28-35, wherein the pharmaceutical composition is for administration selected from the group consisting of airway, oral, ocular, parenteral (including intraveneous, intradermal, intramuscular, intraperitoneal, and subcutaneous), rectal, vaginal and topical (including buccal and sublingual) administration.

37. The use of any one of claims 28-36, wherein the pharmaceutical composition is for use in combination with one or more treatments of disorders selected from the group consisting of treatments for cancer, dermatological disorders, immune
disorders, metabolic disorders, neurological disorders, ocular diseases, infections, and other hereditary and non-hereditary disorders in a mammal.

38. The use of any one of claims 28-37, wherein the mammal is human.

39. An in vitro method of inducing IFN-α production by a mammalian cell, comprising the steps of:
(a) complexing the RNA oligonucleotide of any one of claims 7-16 with a complexation agent; and
(b) contacting the cell with the complex prepared in step (a).

40. The method of claim 39, wherein the mammalian cell is selected from the group consisting of freshly isolated PBMC, freshly isolated PDC, and a cell line capable of producing IFN-α.

41. The method of claim 39 or 40, wherein the complexation agent is a polycationic peptide, preferably poly-L-Arg.

42. An in vitro method of activating mammalian dendritic cells comprising the steps of:
(a) complexing the RNA oligonucleotide of any one of claims 9-20 with a complexation agent;
(b) contacting dendritic cells isolated from a donor mammal with the complexed RNA oligonucleotide; and
(c) contacting the dendritic cells with an antigen.

43. Use of the in vitro activated dendritic cells prepared according to the method of claim 42 for the preparation of a medicament for inducing an immune response in a mammal, wherein the in vitro activated dendritic cells are for transfer into a recipient that is the same or different from the donor.
Fig. 4

- **B2**
- Black bars: Group 1 oligonucleotides
- White bars: Group 2 oligonucleotides

Bars represent the following combinations:
- A*A
- A*C
- A*G
- A*U
- C*A
- C*C
- C*G
- C*U
- G*A
- G*C
- G*G
- G*U
- U*A
- U*C
- U*G
- U*U
Fig. 4

Group 1 oligonucleotides

Group 2 oligonucleotides
Fig. 4
Fig. 4
Fig. 4
Fig. 4
Fig. 4

C12

- Group 1 oligonucleotides
- Group 2 oligonucleotides

Legend:

- UA*A
- UA*C
- UA*G
- UA*U
- UC*A
- UC*C
- UC*G
- UC*U
- UG*A
- UG*C
- UG*G
- UG*U
- UU*A
- UU*C
- UU*G
- UU*U
Fig. 5
Predictive value of 1mer motif 5'-X-3'

Correlation coefficient: + 0.52

Fig. 6
Predictive value of 2mer motif 5'-XX-3'

Correlation coefficient: + 0.77

Fig. 6
Predictive value of 2mer motif 5'-X*X-3'

Correlation coefficient: +0.58

Fig. 6
Predictive value of 2mer motif 5'-X**X-3'

Correlation coefficient: +0.60

Fig. 6
Predictive value of 3mer motif 5'-XXX-3'

Correlation coefficient: +0.87

Fig. 6
Predictive value of 3mer motif 5'-XX*X-3',

Correlation coefficient: +0.80

Fig. 6
Predictive value of 3mer motif 5′-X*XX-3′

Correlation coefficient: + 0.80

Fig. 6
Predictive value of 3mer motif point score

Correlation coefficient: +0.87
IFN-α production [normalized to 9.2sense]

Fig. 7
Fig. 9

A 1mer motifs

5'→U→3'

5'→G→3'

5'→C→3'

5'→A→3'

[IFN-α - % of control]

B 2mer motifs

5'→UU→3'

5'→GU→3'

5'→CU→3'

5'→AU→3'

[IFN-α - % of control]

5'→UG→3'

5'→UC→3'

5'→UA→3'

[IFN-α - % of control]
Fig. 9
<table>
<thead>
<tr>
<th>ANN</th>
<th>AN-N</th>
<th>A-NN</th>
<th>CNN</th>
<th>CN-N</th>
<th>C-NN</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAA</td>
<td>AA-A</td>
<td>A-AA</td>
<td>CA</td>
<td>CA-A</td>
<td>C-AA</td>
</tr>
<tr>
<td>AAC</td>
<td>AA-C</td>
<td>*A-AC</td>
<td>CA</td>
<td>CA-C</td>
<td>C-AC</td>
</tr>
<tr>
<td>AAC</td>
<td>AA-G</td>
<td>*A-AG</td>
<td>CA</td>
<td>CA-G</td>
<td>C-AG</td>
</tr>
<tr>
<td>AAG</td>
<td>AA-U</td>
<td>A-AU</td>
<td>CA</td>
<td>CA-U</td>
<td>C-AU</td>
</tr>
<tr>
<td>ACA</td>
<td>AC-A</td>
<td>*A-CA</td>
<td>CC</td>
<td>CC-A</td>
<td>C-CA</td>
</tr>
<tr>
<td>ACC</td>
<td>AC-C</td>
<td>*A-CC</td>
<td>CC</td>
<td>CC-C</td>
<td>C-CC</td>
</tr>
<tr>
<td>ACG</td>
<td>AC-G</td>
<td>*A-CG</td>
<td>CC</td>
<td>CC-G</td>
<td>C-CG</td>
</tr>
<tr>
<td>AUC</td>
<td>AC-U</td>
<td>A-CU</td>
<td>CC</td>
<td>CC-U</td>
<td>C-CU</td>
</tr>
<tr>
<td>AGA</td>
<td>AG-A</td>
<td>*A-GA</td>
<td>CG</td>
<td>CG-A</td>
<td>C-GA</td>
</tr>
<tr>
<td>AGC</td>
<td>AG-C</td>
<td>*A-GC</td>
<td>CG</td>
<td>CG-C</td>
<td>C-GC</td>
</tr>
<tr>
<td>AGG</td>
<td>AG-G</td>
<td>*A-GG</td>
<td>CG</td>
<td>CG-G</td>
<td>C-GG</td>
</tr>
<tr>
<td>*AGU</td>
<td>AG-U</td>
<td>*A-GU</td>
<td>CG</td>
<td>CG-U</td>
<td>C-GU</td>
</tr>
<tr>
<td>AUG</td>
<td>AU-A</td>
<td>A-UA</td>
<td>CU</td>
<td>CU-A</td>
<td>C-UA</td>
</tr>
<tr>
<td>AUG</td>
<td>AU-C</td>
<td>A-UC</td>
<td>CU</td>
<td>CU-C</td>
<td>C-UC</td>
</tr>
<tr>
<td>AUG</td>
<td>AU-G</td>
<td>A-UG</td>
<td>CU</td>
<td>CU-G</td>
<td>C-UG</td>
</tr>
<tr>
<td>AUU</td>
<td>*AU-U</td>
<td>*A-UU</td>
<td>CU</td>
<td>CU-U</td>
<td>C-UU</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GNN</th>
<th>GN-N</th>
<th>G-NN</th>
<th>UNN</th>
<th>UN-N</th>
<th>U-NN</th>
</tr>
</thead>
<tbody>
<tr>
<td>*GAA</td>
<td>GA-A</td>
<td>G-AA</td>
<td>UA</td>
<td>UA-A</td>
<td>*U-AA</td>
</tr>
<tr>
<td>GAC</td>
<td>GA-C</td>
<td>G-AC</td>
<td>UA</td>
<td>UA-C</td>
<td>U-AC</td>
</tr>
<tr>
<td>GAG</td>
<td>GA-G</td>
<td>G-AG</td>
<td>UA</td>
<td>UA-G</td>
<td>U-AG</td>
</tr>
<tr>
<td>GAU</td>
<td>GA-U</td>
<td>G-AU</td>
<td>UA</td>
<td>UA-U</td>
<td>U-AU</td>
</tr>
<tr>
<td>GCA</td>
<td>GC-A</td>
<td>G-CA</td>
<td>UC</td>
<td>UC-A</td>
<td>*U-CA</td>
</tr>
<tr>
<td>GCC</td>
<td>GC-C</td>
<td>G-CC</td>
<td>UC</td>
<td>UC-C</td>
<td>U-CC</td>
</tr>
<tr>
<td>GCC</td>
<td>GC-G</td>
<td>G-CG</td>
<td>UC</td>
<td>UC-G</td>
<td>U-CG</td>
</tr>
<tr>
<td>GCU</td>
<td>GC-U</td>
<td>G-CU</td>
<td>UC</td>
<td>UC-U</td>
<td>U-CU</td>
</tr>
<tr>
<td>GGA</td>
<td>GG-A</td>
<td>G-GA</td>
<td>UG</td>
<td>UG-A</td>
<td>U-GA</td>
</tr>
<tr>
<td>GGC</td>
<td>GG-C</td>
<td>G-GC</td>
<td>UG</td>
<td>UG-C</td>
<td>U-GC</td>
</tr>
<tr>
<td>GGG</td>
<td>GG-G</td>
<td>G-GG</td>
<td>UG</td>
<td>UG-G</td>
<td>U-GG</td>
</tr>
<tr>
<td>GGU</td>
<td>GG-U</td>
<td>G-GU</td>
<td>UG</td>
<td>UG-U</td>
<td>*U-GU</td>
</tr>
<tr>
<td>*GUA</td>
<td>GU-A</td>
<td>G-UA</td>
<td>UU</td>
<td>UU-A</td>
<td>U-UA</td>
</tr>
<tr>
<td>*GUC</td>
<td>GU-C</td>
<td>*G-UC</td>
<td>UU</td>
<td>UU-C</td>
<td>U-UC</td>
</tr>
<tr>
<td>*GUG</td>
<td>GU-G</td>
<td>G-UG</td>
<td>UU</td>
<td>UU-G</td>
<td>U-UG</td>
</tr>
<tr>
<td>*GUU</td>
<td>GU-U</td>
<td>*G-UU</td>
<td>UU</td>
<td>UU-U</td>
<td>U-UU</td>
</tr>
</tbody>
</table>

Fig. 12
Alphabetical Index for homo sapiens mRNA

1 2 3 4 5 6 7 8 A B C D E F G H I J K L M N O P Q R S T U V

W X Y Z

Fig. 19
B Predicted IFN-α induction for siRNAs targeting hs VEGF transcript variant 1, mRNA

Accession number (Name of the RNA transcript)

Gene # 3248 : NM_001025366.1 (Homo sapiens vascular endothelial growth factor (VEGF), transcript variant 1, mRNA).

Graphical depiction of the mRNA:
- Both siRNA strands above the threshold
- Antisense strand below the threshold
- Sense strand below the threshold
- Both strands below the threshold

List of sequences that are below the threshold:
- Position (starting base of the respective sense strand / total number of bases)
- Value (predicted IFN-α induction level for the strand below the threshold)
- Sequence (base sequence of the sense strand)
- Indication whether the SENSE, the ANTISENSE or BOTH strand are below the threshold

Fig. 18
<110> Hartmann, Gunther

<120> Compositions and methods for immunostimulatory RNA oligonucleotides

<130> PCT44708

<150> EP 05020002.3
<151> 2005-14-09

<150> EP 05027778.9
<151> 2005-19-12

<150> US 60/717, 323
<151> 2005-15-09

<160> 390

<170> PatentIN version 3.3

<210> 1
<211> 19
<212> RNA
<213> Artificial

<220> .
<223> randomized 4 mer motifs on poly A backbone

<400> 1
aaaaaaaaaa aaaaaaaaa 19

<210> 2
<211> 19
<212> RNA
<213> Artificial

<220> .
<223> randomized 4 mer motifs on poly A backbone

<400> 2
aaaaaaacaa aaaaaaaaa 19

<210> 3
<211> 19
<212> RNA
<213> Artificial

<220> .
<223> randomized 4 mer motifs on poly A backbone

<400> 3
aaaaaaaaga aaaaaaaaa 19

<210> 4
<211> 19
<212> RNA
<213> Artificial

<220> .
<223> randomized 4 mer motifs on poly A backbone

<400> 4
aaaaaaaauat aaaaaaaaa 19
<210> 5
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 5
aaaaaaaacc aaaaaaaa 19

<210> 6
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 6
aaaaaaacg aaaaaaaaa 19

<210> 7
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 7
aaaaaaaacu aaaaaaaa 19

<210> 8
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 8
aaaaaaagc aaaaaaaaa 19

<210> 9
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 9
aaaaaaaggg aaaaaaaa 19

<210> 10
<211> 19
<212> RNA
<213> Artificial

<220>
randomized 4 mer motifs on poly A backbone

<200> 10
aaaaaaaagu aaaaaaaaa 19
<210> 11
<211> 19
<212> RNA
<213> Artificial
<220> randomized 4 mer motifs on poly A backbone
<223> randomized 4 mer motifs on poly A backbone
<400> 11
aaaaaaaauc aaaaaaaaa 19
<210> 12
<211> 19
<212> RNA
<213> Artificial
<220> randomized 4 mer motifs on poly A backbone
<400> 12
aaaaaaaaug aaaaaaaaa 19
<210> 13
<211> 19
<212> RNA
<213> Artificial
<220> randomized 4 mer motifs on poly A backbone
<400> 13
aaaaaaaauu aaaaaaaaa 19
<210> 14
<211> 19
<212> RNA
<213> Artificial
<220> randomized 4 mer motifs on poly A backbone
<400> 14
aaaaaaaaca caaaaaaaaa 19
<210> 15
<211> 19
<212> RNA
<213> Artificial
<220> randomized 4 mer motifs on poly A backbone
<400> 15
aaaaaaaaca gaaaaaaaa 19
<210> 16
<211> 19

Seite 3
RNA Artificial
randomized 4 mer motifs on poly A backbone

16 aaaaaaaaaaca uaaaaaaaaa 19

17 aaaaaaaaaacc caaaaaaaaa 19

18 aaaaaaaaaacc gaaaaaaaaa 19

19 aaaaaaaaaacc gaaaaaaaaa 19

20 aaaaaaaaaacc uaaaaaaaaa 19

21 aaaaaaaaaacg caaaaaaaaa 19

21 aaaaaaaaaacg gaaaaaaaaa 19
Artificial randomized 4 mer motifs on poly A backbone

```
<400> 22
aaaaaaaacg uaaaaaaaa 19
```

```
<400> 23
aaaaaaacu caaaaaaaaa 19
```

```
<400> 24
aaaaaaacu caaaaaaaaa 19
```

```
<400> 25
aaaaaaacu uaaaaaaaa 19
```

```
<400> 26
aaaaaaaga caaaaaaaaa 19
```

```
<400> 27
aaaaaaaga caaaaaaaaa 19
```
randomized 4 mer motifs on poly A backbone

<table>
<thead>
<tr>
<th>Number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>aaaaaaaaaaga</td>
</tr>
<tr>
<td>28</td>
<td>aaaaaaaaaaga</td>
</tr>
<tr>
<td>29</td>
<td>aaaaaaaaaaga</td>
</tr>
<tr>
<td>30</td>
<td>aaaaaaaaaaga</td>
</tr>
<tr>
<td>31</td>
<td>aaaaaaaaaaga</td>
</tr>
<tr>
<td>32</td>
<td>aaaaaaaaaaga</td>
</tr>
<tr>
<td>33</td>
<td>aaaaaaaaaaga</td>
</tr>
</tbody>
</table>
RNA Artificial

randomized 4 mer motifs on poly A backbone

aaaaaaag 19

aaaaaaag 19

aaaaaaaga 19

aaaaaaagu 19

aaaaaaagu 19

aaaaaaagu 19

aaaaaaagu 19

aaaaaaagu 19
PCT4708SeqList.txt

<210> 39
<211> 19
<212> RNA
<213> Artificial

<220>
<223> randomized 4 mer motifs on poly A backbone

<400> 39
aaaaaaaaua gaaaaaaaa 19

<210> 40
<211> 19
<212> RNA
<213> Artificial

<220>
<223> randomized 4 mer motifs on poly A backbone

<400> 40
aaaaaaaaua uaaaaaaaa 19

<210> 41
<211> 19
<212> RNA
<213> Artificial

<220>
<223> randomized 4 mer motifs on poly A backbone

<400> 41
aaaaaaaauc caaaaaaaaa 19

<210> 42
<211> 19
<212> RNA
<213> Artificial

<220>
<223> randomized 4 mer motifs on poly A backbone

<400> 42
aaaaaaaauc gaaaaaaaa 19

<210> 43
<211> 19
<212> RNA
<213> Artificial

<220>
<223> randomized 4 mer motifs on poly A backbone

<400> 43
aaaaaaaauc uaaaaaaaa 19

<210> 44
<211> 19
<212> RNA
<213> Artificial

<220>

Seite 8
randomized 4 mer motifs on poly A backbone

44
aaaaaaaaug caaaaaaa 19

45
<211> 19
<212> RNA
<213> Artificial

46
aaaaaaaaaug gaaaaaaa 19

47
<211> 19
<212> RNA
<213> Artificial

48
aaaaaaaaauu uaaaaaaa 19

49
<211> 19
<212> RNA
<213> Artificial

49
aaaaaaaaauu uaaaaaaa 19

50
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 50
aaaaaaacaa caaaaaaaa  19

<210> 51
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 51
aaaaaaacaa gaaaaaaaa  19

<210> 52
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 52
aaaaaaacaa uaaaaaaaa  19

<210> 53
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 53
aaaaaaacac caaaaaaaa  19

<210> 54
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 54
aaaaaaacac gaaaaaaaa  19

<210> 55
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 55
aaaaaaacac uaaaaaaaa  19
Randomized 4 mer motifs on poly A backbone

aaaaaaacag caaaaaaa

Randomized 4 mer motifs on poly A backbone

aaaaaaacag gaaaaaaa

Randomized 4 mer motifs on poly A backbone

aaaaaaacag uaaaaaaa

Randomized 4 mer motifs on poly A backbone

aaaaaaacau caaaaaaa

Randomized 4 mer motifs on poly A backbone

aaaaaaacau gaaaaaaa

Randomized 4 mer motifs on poly A backbone

aaaaaaacau gaaaaaaa
randomized 4 mer motifs on poly A backbone

<400> 61
aaaaaaacau uaaaaaaaa

<210> 62
<211> 19
<212> RNA
<213> Artificial

<223> randomized 4 mer motifs on poly A backbone

<400> 62
aaaaaaacca caaaaaaa

<210> 63
<211> 19
<212> RNA
<213> Artificial

<223> randomized 4 mer motifs on poly A backbone

<400> 63
aaaaaaacca gaaaaaaa

<210> 64
<211> 19
<212> RNA
<213> Artificial

<223> randomized 4 mer motifs on poly A backbone

<400> 64
aaaaaaacca uaaaaaaaa

<210> 65
<211> 19
<212> RNA
<213> Artificial

<223> randomized 4 mer motifs on poly A backbone

<400> 65
aaaaaaaccc caaaaaaa

<210> 66
<211> 19
<212> RNA
<213> Artificial

<223> randomized 4 mer motifs on poly A backbone

<400> 66
aaaaaaaccc gaaaaaaaa

<210> 67
<211> 19
RNA Artificial

randomized 4 mer motifs on poly A backbone

aaaaaaaccu uaaaaaaaaa 19

aaaaaaaccg caaaaaaa 19

aaaaaaaccg gaaaaaaaa 19

aaaaaaaccg gaaaaaaaa 19

aaaaaaaccu uaaaaaaaaa 19

aaaaaaaccu gaaaaaaa 19

aaaaaaaccu caaaaaaa 19

aaaaaaaccu gaaaaaaa 19
<210> 73
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 73
aaaaaaaccu uaaaaaaaaa 19

<210> 74
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 74
aaaaaaacga caaaaaaa 19

<210> 75
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 75
aaaaaaacga caaaaaaa 19

<210> 76
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 76
aaaaaaacga uaaaaaaa 19

<210> 77
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 77
aaaaaaacgc caaaaaaa 19

<210> 78
<211> 19
<212> RNA
<213> Artificial

<220>
randomized 4 mer motifs on poly A backbone

78
aaaaaagcg gaaaaaaaa

79
aaaaaagcg uaaaaaaaa

79
aaaaaagcg uaaaaaaaa

80
aaaaaaagcg caaaaaaaaa

80
aaaaaaagcg caaaaaaaaa

81
aaaaaaagcg caaaaaaaaa

81
aaaaaaagcg caaaaaaaaa

82
aaaaaaagcg caaaaaaaaa

82
aaaaaaagcg caaaaaaaaa

83
aaaaaaagcg caaaaaaaaa

83
aaaaaaacgu caaaaaaaaa

84

RNA Artificial

randomized 4 mer motifs on poly A backbone

84
aaaaaacgu gaaaaaaaa

19

85
<210> 85
<211> 19
<212> RNA
<213> Artificial

randomized 4 mer motifs on poly A backbone

85
aaaaaacgu uaaaaaaa

19

86
<210> 86
<211> 19
<212> RNA
<213> Artificial

randomized 4 mer motifs on poly A backbone

86
aaaaaacua caaaaaaa

19

87
<210> 87
<211> 19
<212> RNA
<213> Artificial

randomized 4 mer motifs on poly A backbone

87
aaaaaacua gaaaaaaa

19

88
<210> 88
<211> 19
<212> RNA
<213> Artificial

randomized 4 mer motifs on poly A backbone

88
aaaaaacua uaaaaaaaa

19

89
<210> 89
<211> 19
<212> RNA
<213> Artificial

randomized 4 mer motifs on poly A backbone

89
aaaaaacuc caaaaaaaaa

19
PCT44708SeqList .txt

<210>  90
<211>  19
<212>  RNA
<213>  Artificial

<220> randomized 4 mer motifs on poly A backbone
<400>  90
aaaaaaacuc gaaaaaaaa

<210>  91
<211>  19
<212>  RNA
<213>  Artificial

<220> randomized 4 mer motifs on poly A backbone
<400>  91
aaaaaaacuc uaaaaaaaa

<210>  92
<211>  19
<212>  RNA
<213>  Artificial

<220> randomized 4 mer motifs on poly A backbone
<400>  92
aaaaaaacug caaaaaaaaa

<210>  93
<211>  19
<212>  RNA
<213>  Artificial

<220> randomized 4 mer motifs on poly A backbone
<400>  93
aaaaaaacug gaaaaaaaa

<210>  94
<211>  19
<212>  RNA
<213>  Artificial

<220> randomized 4 mer motifs on poly A backbone
<400>  94
aaaaaaacug uaaaaaaaa

<210>  95
<211>  19
<212>  RNA
<213>  Artificial

<220>

Seite 17
randomized 4 mer motifs on poly A backbone

<400> 95
aaaaaaacuu caaaaaaaa 19

<210> 96
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 96
aaaaaaacuu gaaaaaaaa 19

<210> 97
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 97
aaaaaaacuu uaaaaaaaa 19

<210> 98
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 98
aaaaaaagaa caaaaaaaa 19

<210> 99
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 99
aaaaaaagaa gaaaaaaaa 19

<210> 100
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 100
aaaaaaagaa uaaaaaaaa 19

<210> 101
<211> 19
RNA Artificial
randomized 4 mer motifs on poly A backbone

<400> 101
aaaaaaagac caaaaaaaaa 19

<210> 102
<211> 19
<212> RNA
<213> Artificial
randomized 4 mer motifs on poly A backbone

<400> 102
aaaaaaagac gaaaaaaaa 19

<210> 103
<211> 19
<212> RNA
<213> Artificial
randomized 4 mer motifs on poly A backbone

<400> 103
aaaaaaagac uaaaaaaaa 19

<210> 104
<211> 19
<212> RNA
<213> Artificial
randomized 4 mer motifs on poly A backbone

<400> 104
aaaaaaagag caaaaaaaaa 19

<210> 105
<211> 19
<212> RNA
<213> Artificial
randomized 4 mer motifs on poly A backbone

<400> 105
aaaaaaagag gaaaaaaaaaa 19

<210> 106
<211> 19
<212> RNA
<213> Artificial
randomized 4 mer motifs on poly A backbone

<400> 106
aaaaaaagag uaaaaaaaaa 19
PCT44708seqList.txt

<210> 107
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 107
aaaaaaagau caaaaaaa 19

<210> 108
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 108
aaaaaaagau gaaaaaaa 19

<210> 109
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 109
aaaaaaagau uaaaaaaaa 19

<210> 110
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 110
aaaaaaagca caaaaaaa 19

<210> 111
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 111
aaaaaaagca gaaaaaaa 19

<210> 112
<211> 19
<212> RNA
<213> Artificial

<220>
randomized 4 tier motifs on poly A backbone

<400> 112
aaaaaaagca uaaaaaaa

<210> 113
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 113
aaaaaaagcc caaaaaaa

<210> 114
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 114
aaaaaaagcc gaaaaaaa

<210> 115
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 115
aaaaaaagcc uaaaaaaa

<210> 116
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 116
aaaaaaagcg caaaaaaa

<210> 117
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 117
aaaaaaagcg gaaaaaaa

<210> 118
<211> 19
<212> RNA
<213> Artificial
<220> randomized 4 mer motifs on poly A backbone
<400> 118
aaaaaagcgu uaaaaaaa 19
<210> 119
<211> 19
<212> RNA
<213> Artificial
<220> randomized 4 mer motifs on poly A backbone
<400> 119
aaaaaagcu caaaaaaa 19
<210> 120
<211> 19
<212> RNA
<213> Artificial
<220> randomized 4 mer motifs on poly A backbone
<400> 120
aaaaaagcu gaaaaaaa 19
<210> 121
<211> 19
<212> RNA
<213> Artificial
<220> randomized 4 mer motifs on poly A backbone
<400> 121
aaaaaagcu uaaaaaaaa 19
<210> 122
<211> 19
<212> RNA
<213> Artificial
<220> randomized 4 mer motifs on poly A backbone
<400> 122
aaaaaaaagga caaaaaaaaa 19
<210> 123
<211> 19
<212> RNA
<213> Artificial
<220> randomized 4 mer motifs on poly A backbone
<400> 123
aaaaaaaagga gaaaaaaaa 19
randomized 4 mer motifs on poly A backbone

```
124
aaaaaaagga uaaaaaaa
```

```
125
aaaaaaaggc caaaaaaa
```

```
126
aaaaaaaggc gaaaaaaa
```

```
127
aaaaaaaggc uaaaaaaa
```

```
128
aaaaaaaggg caaaaaaa
```

```
129
aaaaaaaggg caaaaaaa
```

Seite 23
<223> randomized 4 mer motifs on poly A backbone

<400>  129
aaaaaaaggg gaaaaaaa

<210>  130
<211>  19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400>  130
aaaaaaaggg uaaaaaaa

<210>  131
<211>  19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400>  131
aaaaaaaggu caaaaaaa

<210>  132
<211>  19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400>  132
aaaaaaaggu gaaaaaaa

<210>  133
<211>  19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400>  133
aaaaaaaggu uaaaaaaa

<210>  134
<211>  19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400>  134
aaaaaaagua caaaaaaa

<210>  135
<211>  19
RNA Artificial
randomized 4 mer motifs on poly A backbone

aaaaaaagua gaaaaaaaaa 19

aaaaaaagua uaaaaaaaa 19

aaaaaaaguc caaaaaaaa 19

aaaaaaaguc caaaaaaaa 19

aaaaaaaguc gaaaaaaaaa 19

aaaaaaaguc gaaaaaaaaa 19

aaaaaagug caaaaaaaa 19

aaaaaagug caaaaaaaa 19
randomized 4 mer motifs on poly A backbone

aaaaaaagug gaaaaaaaa

19

randomized 4 mer motifs on poly A backbone

aaaaaaagug uaaaaaaaa

19

randomized 4 mer motifs on poly A backbone

aaaaaaaguu caaaaaaaa

19

randomized 4 mer motifs on poly A backbone

aaaaaaaguu gaaaaaaaa

19

randomized 4 mer motifs on poly A backbone

aaaaaaaguu uaaaaaaaa

19

randomized 4 mer motifs on poly A backbone

aaaaaaaguu uaaaaaaaa

19
randomized 4 mer motifs on poly A backbone

aaaaaaauaa caaaaaaaa 19

aaaaaaauaa gaaaaaaa 19

aaaaaaauaa uaaaaaaa 19

aaaaaaauac caaaaaaa 19

aaaaaaauac gaaaaaaa 19

aaaaaaauac uaaaaaaa 19
RNA Artificial

randomized 4 mer motifs on poly A backbone

aaaaaaauag caaaaaaaa 19

aaaaaaauag gaaaaaaaa 19

aaaaaaauag uaaaaaaa 19

aaaaaaauau caaaaaaaa 19

aaaaaaauau uaaaaaaa 19

aaaaaaauau uaaaaaaa 19

aaaaaaauau uaaaaaaa 19
<210> 158
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 158
 aaaaaauca caaaaaaa

<210> 159
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 159
 aaaaaauca gaaaaaaa

<210> 160
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 160
 aaaaaauca uaaaaaaaa

<210> 161
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 161
 aaaaaaaucc caaaaaaaa

<210> 162
<211> 19
<212> RNA
<213> Artificial

<220> randomized 4 mer motifs on poly A backbone

<400> 162
 aaaaaaaucc gaaaaaaaa

<210> 163
<211> 19
<212> RNA
<213> Artificial

<220>
randomized 4 mer motifs on poly A backbone

aaaaaucc uaaaaaaaa 19

aaaaaaucg caaaaaaaaa 19

aaaaaaucg gaaaaaaaaa 19

aaaaaaucg uaaaaaaaa 19

aaaaaaucu caaaaaaaaa 19

aaaaaaucu gaaaaaaa 19

Seite 30
RNA Artificial

randomized 4 mer motifs on poly A backbone

aaaaaaaucu uaaaaaaaaa

19

aaaaaauga caaaaaaaaa

19

aaaaaauga gaaaaaaaaa

19

aaaaaauga uaaaaaaaaa

19

aaaaaaaugc caaaaaaaaa

19

aaaaaaaugc gaaaaaaaaa

19
randomized 4 mer motifs on poly A backbone

aaaaaaaaugg uaaaaaaaa 19

randomized 4 mer motifs on poly A backbone

aaaaaaaaugg gaaaaaaaa 19

randomized 4 mer motifs on poly A backbone

aaaaaaaaugg gaaaaaaaa 19

randomized 4 mer motifs on poly A backbone

aaaaaaaaugg uaaaaaaaa 19

randomized 4 mer motifs on poly A backbone

aaaaaaaaaugu caaaaaaaa 19

randomized 4 mer motifs on poly A backbone

aaaaaaaaaugc uaaaaaaaa 19
randomized 4 mer motifs on poly A backbone

aaaaaaaugu gaaaaaaa 19

aaaaaaaugu uaaaaaa 19

aaaaaauua caaaaaaa 19

aaaaaauua gaaaaaaa 19

aaaaaaauua caaaaaaa 19

aaaaaaauua uaaaaaaa 19

aaaaaaauua uaaaaaaa 19

aaaaaaauuc caaaaaaa 19

aaaaaaauuc caaaaaaa 19

aaaaaaauuc caaaaaaa 19


RNA Artificial
randomized 4 mer motifs on poly A backbone

186
aaaaaaauuc gaaaaaaa 19

187
19

RNA Artificial
randomized 4 mer motifs on poly A backbone

187
aaaaaaauuc uaaaaaaa 19

188
19

RNA Artificial
randomized 4 mer motifs on poly A backbone

188
aaaaaaauug caaaaaaaa 19

189
19

RNA Artificial
randomized 4 mer motifs on poly A backbone

189
aaaaaaauug gaaaaaaa 19

190
19

RNA Artificial
randomized 4 mer motifs on poly A backbone

190
aaaaaaauug uaaaaaaa 19

191
19

RNA Artificial
randomized 4 mer motifs on poly A backbone

191
aaaaaaauuu caaaaaaa 19
randomized 4 mer motifs on poly A backbone

AAAAAAUUUU

different positions of the GUCA motif on poly A backbone

AAGUCAAAAA

different positions of the GUCA motif on poly A backbone

AAAAAAGUCA

different positions of the GUCA motif on poly A backbone

AAAAAAGUCA
different positions of the GUCA motif on poly A backbone

aaaaaaaaaa gucaaaaaa

197

<210> 198
<211> 19
<212> RNA
<213> Artificial

<220>
different positions of the GUCA motif on poly A backbone

aaaaaaaaaa aaaaaguca

198

19

<212> RNA
<213> Artificial

<220>
single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

aaaaaaaaaaagu caaaaaaa

199

19

<212> RNA
<213> Artificial

<220>
single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

aaaaaaaaaaagu cacaaaaa

200

19

<212> RNA
<213> Artificial

<220>
single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

aaaaaaaaaaagu cauaaaaa

201

19

<212> RNA
<213> Artificial

<220>
single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

aaaaaaaaaaagu caaaaaaa

202

19

<212> RNA
<213> Artificial

<220>
single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

aaaaaaaaaaagu cauaaaaa

202

19
<210> 203
<211> 19
<212> RNA
<213> Artificial

<220> single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

<400> 203
aaaaaaacgu caaaaaaaa 19

<210> 204
<211> 19
<212> RNA
<213> Artificial

<220> single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

<400> 204
aaaaaaacgu cacaaaaaa 19

<210> 205
<211> 19
<212> RNA
<213> Artificial

<220> single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

<400> 205
aaaaaaacgu cagaaaaaa 19

<210> 206
<211> 19
<212> RNA
<213> Artificial

<220> single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

<400> 206
aaaaaaacgu caaaaaaa 19

<210> 207
<211> 19
<212> RNA
<213> Artificial

<220> single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

<400> 207
aaaaaaaggu caaaaaaa 19

<210> 208
Artificial

single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

aaaaaaaggu cacaaaaaa

aaaaaaaggu cagaaaaaa

aaaaaaaggu cauaaaaaa

aaaaaaaugu cacaaaaaa

aaaaaaaugu caaaaaaaaaa
single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

aaaaaaugu cagaaaaa 19

RNA Artificial

single base permutations to the 5' and the 3' end of GUCA motif on poly A backbone

aaaaaaugu caaaaaaa 19

RNA Artificial

sense strand of siRNA specific for human TLR9 with the starting base 1647 (siRNA9.2)

agcuuaccu guccuuaa 19

RNA Artificial

poly A modification of the sense strand of siRNA specific for human TLR9 with the starting base 1647 (siRNA9.2)

aaaaaaccu guccuuc 19

RNA Artificial

poly A modification of the sense strand of siRNA specific for human TLR9 with the starting base 1647 (siRNA9.2)

aaaaaaacu guccuuc 19

RNA Artificial

poly A modification of the sense strand of siRNA specific for
human TLR9 with the starting base 1647 (siRNA9.2)

<table>
<thead>
<tr>
<th>&lt;400&gt;</th>
<th>218</th>
</tr>
</thead>
<tbody>
<tr>
<td>aaaaaaaaaa guccuucaa</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>&lt;220&gt;</th>
<th>poly A modification of the sense strand of siRNA specific for</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;223&gt;</td>
<td>human TLR9 with the starting base 1647 (siRNA9.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>&lt;400&gt;</th>
<th>220</th>
</tr>
</thead>
<tbody>
<tr>
<td>aaaaaaaaaa auccuucaa</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>&lt;220&gt;</th>
<th>poly A modification of the sense strand of siRNA specific for</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;223&gt;</td>
<td>human TLR9 with the starting base 1647 (siRNA9.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>&lt;400&gt;</th>
<th>221</th>
</tr>
</thead>
<tbody>
<tr>
<td>aaaaaaaaaa aacuuucca</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>&lt;220&gt;</th>
<th>poly A modification of the sense strand of siRNA specific for</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;223&gt;</td>
<td>human TLR9 with the starting base 1647 (siRNA9.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>&lt;400&gt;</th>
<th>222</th>
</tr>
</thead>
<tbody>
<tr>
<td>agcuaaccu aaaaaaaaaa</td>
<td>19</td>
</tr>
</tbody>
</table>
agcuuaaccu  gaaaaaaaa 1 9
<210> 224
<211> 19
<212> RNA
<213> Artificial

<220>
<223> poly A modification of the sense strand of siRNA specific for human TLR9 with the starting base 1647 (siRNA9.2)

<400> 224
agcuuaaccu guaaaaaa 19

<210> 225
<211> 19
<212> RNA
<213> Artificial

<220>
<223> poly A modification of the sense strand of siRNA specific for human TLR9 with the starting base 1647 (siRNA9.2)

<400> 225
agcuuaaccu guacaaaaaa 19

<210> 226
<211> 19
<212> RNA
<213> Artificial

<220>
<223> poly A modification of the sense strand of siRNA specific for human TLR9 with the starting base 1647 (siRNA9.2)

<400> 226
agcuuaaccu guccaaaaaa 19

<210> 227
<211> 19
<212> RNA
<213> Artificial

<220>
<223> poly A modification of the sense strand of siRNA specific for human TLR9 with the starting base 1647 (siRNA9.2)

<400> 227
agcuuaaccu guccuuaaa 19

<210> 228
<211> 19
<212> RNA
<213> Artificial

<220>
<223> poly A modification of the sense strand of siRNA specific for human TLR9 with the starting base 1647 (siRNA9.2)

<400> 228
agcuuaaccu guccuuaaa 19
SSRNA oligonucleotide "RNA40" (Heil et al.)

```plaintext
gccgucugu ugugacuc
```

sense strand of siRNA specific for human beta-Gal actosidase (Judge et al.)

```plaintext
uugauguguu uagucgcua
```

sense strand of siRNA specific for human TLR9 with the starting base 271

```plaintext
caucucuccc ugcucucug
```

anti-sense strand of siRNA specific for human TLR9 with the starting base 271

```plaintext
cagagagcag ggagagaug
```

sense strand of siRNA specific for human TLR9 with the starting base 1122

```plaintext
ucgccggccu gcaugcccu
```

anti-sense strand of siRNA specific for human TLR9 with the starting base 1122

```plaintext
cagagagcag ggagagaug
```
antisense strand of siRNA specific for human TLR9 with the starting base 1122

aggcaugca ggccggcga

sense strand of siRNA specific for human TLR9 with the starting base 1949

gaggcaug gagggaga

antisense strand of siRNA specific for human TLR9 with the starting base 1302

ucuccuccuc aucugccuc

sense strand of siRNA specific for human TLR9 with the starting base 1302

aguauugcu guuguccua

antisense strand of siRNA specific for human TLR9 with the starting base 1302

uaggcaaca gcagauacu
base 1500

<400> 239
  ugguguugaa ggacaguuc 19
<210> 240
<211> 19
<212> RNA
<213> Artificial
<220>
<223> anti-sense strand of siRNA specific for human TLR9 with the starting base 1500

<400> 240
gaacugucc ucaacacca 19
<210> 241
<211> 19
<212> RNA
<213> Artificial
<220>
<223> sense strand of siRNA specific for human TLR9 with the starting base 1019

<400> 241
  aaccugagcu acaacaaca 19
<210> 242
<211> 19
<212> RNA
<213> Artificial
<220>
<223> anti-sense strand of siRNA specific for human TLR9 with the starting base 1019

<400> 242
  uguuguugua gcucagguu 19
<210> 243
<211> 21
<212> RNA
<213> Artificial
<220>
<223> example ssRNA oligonucleotide (hairpin sequence)

<400> 243
gaccuagccu aaaaacuaggu c 21
<210> 244
<211> 17
<212> RNA
<213> Artificial
<220>
<223> example ssRNA oligonucleotide (palindromic sequence)

<400> 244
  aaagauccgg aucaaaa 17
example ssRNA oligonucleotide (partially double stranded sequence 1 example 1)

aaaaguucga agcucaaa

example ssRNA oligonucleotide (partially double stranded sequence 2 example 1)

cagguuucga g

example ssRNA oligonucleotide (partially double stranded sequence 1 example 2)

ucaaagucua aagcucaag uugaaaag uaa

example ssRNA oligonucleotide (partially double stranded sequence 2 example 2)

gacuugaaa uuucaguuuu cgaguuuag uugaaacuc g

example ssRNA oligonucleotide (partially double stranded sequence 1 example 3)

ucaaagucua aagcucaag uugaa

example ssRNA oligonucleotide (partially double stranded sequence 1 example 4)

ucaaagucua aagcucaag uuga
<table>
<thead>
<tr>
<th>Line</th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>uuucaguuuu cgaguuaag uugaaacuc g</td>
<td>31</td>
</tr>
<tr>
<td>251</td>
<td>cagagcggga ugcguugguc</td>
<td>20</td>
</tr>
<tr>
<td>252</td>
<td>uugauguguu uagucgcu</td>
<td>19</td>
</tr>
<tr>
<td>253</td>
<td>gcaccacuag uugguuguc</td>
<td>19</td>
</tr>
<tr>
<td>254</td>
<td>guuguaguug uacuccagc</td>
<td>19</td>
</tr>
<tr>
<td>255</td>
<td>guuguaguug uacuccagc</td>
<td>19</td>
</tr>
</tbody>
</table>
<400> 255
gccgcgucugu ugugugacuc 20

<210> 256
<211> 12
<212> RNA
<213> Artificial

<220>
<223> disclaimer SSNA oligonucleotide "RNA40" (Heil et al. 2)

<400> 256
gucuguugug ug 12

<210> 257
<211> 18
<212> RNA
<213> Artificial

<220>
<223> disclaimer ssRNA oligonucleotide C Patent WO 03/086280

<400> 257
guuguguug ugguugug 18

<210> 258
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example SSNA oligonucleotide (motif in different positions)

<400> 258
aaaaaaaguu caaaaaaa 19

<210> 259
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example SSNA oligonucleotide (motif in different positions)

<400> 259
aaaguucaaa aaaaaaaaa 19

<210> 260
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example SSNA oligonucleotide (motif in different positions)

<400> 260
aaaaaaaaaa aaguucaaa 19

<210> 261
<211> 19
<212> RNA
<213> Artificial
example ssRNA oligonucleotide (more than one motif)

```
<400> 261
aaaguucaaa aaguucaaa
```

RNA Artificial

element ssRNA oligonucleotide (more than one motif)

```
<400> 262
guucaaaaguu caaaguuca
```

RNA Artificial

element ssRNA oligonucleotide (different motifs)

```
<400> 263
aguucaaga guaagucuc
```

RNA Artificial

element ssRNA oligonucleotide (different motifs)

```
<400> 264
aguucaguuc aagucaagc uc
```

RNA Artificial

element ssRNA oligonucleotide (different motifs)

```
<400> 265
aaaguucaaa gucaaaagcu caaaguugaa aguuaaagg uuaaa
```

RNA Artificial

element ssRNA oligonucleotide (overlapping motifs)

```
<400> 266
aaaaguugcu caaaaaa
```

Seite 48
PCT44708SeqList.txt

<210> 267
<211> 10
<212> RNA
<213> Artificial

<22O>
<223> ssRNA oligonucleotide "RNA33" (Heil et al.)

<400> 267
guagugugug 10

<210> 268
<211> 21
<212> RNA
<213> Artificial

<22O>
<223> ssRNA oligonucleotide "sense 27" (Sioud et al.)

<400> 268
guccggcag gucuacuuu u 21

<210> 269
<211> 21
<212> RNA
<213> Artificial

<22O>
<223> ssRNA oligonucleotide "sense 32" (Sioud et al.)

<400> 269
gcuggagauc cugaagaacu u 21

<210> 270
<211> 19
<212> RNA
<213> Artificial

<22O>
<223> ssRNA oligonucleotide "beta-Gal control sense" (Judge et al.)

<400> 270
uugauguguu uagucguua 19

<210> 271
<211> 19
<212> RNA
<213> Artificial

<22O>
<223> ssRNA oligonucleotide "beta-Gal control antisense" (Judge et al.)

<400> 271
uagcaguaa acacauca 19

<210> 272
<211> 19
<212> RNA
<213> Artificial

<22O>
<223> ssRNA oligonucleotide "5' UTR" (Barchet et al.)
<400> 272
aguagaaaca agguaguuu

<210> 273
<211> 19
<212> RNA
<213> Artificial

<220>
<223> ssRNA oligonucleotide "3' UTR" (Barchet et al.)

<400> 273
uuaaucuaccu gcuuuugcu

<210> 274
<211> 42
<212> RNA
<213> Artificial

<220>
<223> ssRNA oligonucleotide "5' 3' UTR" (Barchet et al.)

<400> 274
aguagaaaca agguaguuuu uuguuaacu ccugcuuuug cu

<210> 275
<211> 19
<212> RNA
<213> Artificial

<220>
<223> ssRNA oligonucleotide "5' UTR U-C replacement" (Barchet et al.)

<400> 275
agcagaaaca aggccagccc

<210> 276
<211> 19
<212> RNA
<213> Artificial

<220>
<223> ssRNA oligonucleotide "5' UTR G-C replacement" (Barchet et al.)

<400> 276
acuacaaaca accuacuuu

<210> 277
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example ssRNA oligonucleotide (Group A oligos - above threshold)

<400> 277
guuuugcu uugauugcc

<210> 278
<211> 19
<212> RNA
<213> Artificial
example ssRNA oligonucleotide (Group A oligos - above threshold)

uuguaguucguugcuagug

19

example ssRNA oligonucleotide (Group A oligos - above threshold)

aguucauggugguuguac

19

example ssRNA oligonucleotide (Group A oligos - above threshold)

uguuuaaguuuguucuaccc

19

example ssRNA oligonucleotide (Group A oligos - above threshold)

aaguuuugauuuucagua

19

example ssRNA oligonucleotide (Group A oligos - above threshold)

aggcguuuguuguucggu

19

example ssRNA oligonucleotide (Group A oligos - above threshold)

agauguuguaugguguuu

19
example ssRNA oligonucleotide (Group A oligos - above threshold)

uagugugugu cagugugac

example ssRNA oligonucleotide (Group A oligos - above threshold)
gguugcgugu ggaguuguu

example ssRNA oligonucleotide (Group A oligos - above threshold)
guaguuuuug uuagaguca

example ssRNA oligonucleotide (Group A oligos - above threshold)
guguguugc uguuguca

example ssRNA oligonucleotide (Group B oligos - below threshold)
gggaccgaaa gaccagacc

example ssRNA oligonucleotide (Group B oligos - below threshold)
uaagacuaga  agagacaga  

agaucgaac  caccgacca  

gaucagaa  auagacag  

cauauaagaa  gaccagcca  

uaagaaccaa  cugcuagaa  

ccccuacaga  cagaauacc  

uaagaaccaa  cuagcuagaa  

ccccuacaga  cagaauacc  

example ssRNA oligonucleotide (Group B oligos - below threshold)

cuggcagaauagauagac 19

cuagaccagaacaauucg 19

RNA Artificial

eample ssRNA oligonucleotide (Group B oligos - below threshold)
uuagagcauaacaauuu 19

gaccaaccucucgacau 19

gaccaaccucucgacau 19

example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - anti sense strand)

uaacaaacucucacca 19

uguugguaggaguuuguua 19

example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - sense strand)

uaacaaacucucacca 19

uguugguaggaguuuguua 19
<210> 301
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)

<400> 301
aacaaacucc uaccaacac

<210> 302
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - antisense strand)

<400> 302
uguuugguag gauuuuguu

<210> 303
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)

<400> 303
uaccaacacu gaccaaa

<210> 304
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - antisense strand)

<400> 304
uuauugguca guguuggu

<210> 305
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)

<400> 305
cuaccaacac uaccaaa

<210> 306
example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - antisense strand)

uuuauugguc aguguuggu 19

example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)

accaacacu accaauaaa 19

example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - antisense strand)

uuuauugguc aguguuggu 19

example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - sense strand)

acaacuccu accaacucu 19

example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - antisense strand)

aguguuggu ggaguuguu 19
<220> example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)

<400> 311
acuccuacca acacugacc 19

<210> 312
<211> 19
<212> RNA
<213> Artificial

<220> example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - antisense strand)

<400> 312
ggucaguguu guuaggag 19

<210> 313
<211> 19
<212> RNA
<213> Artificial

<220> example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - sense strand)

<400> 313
cuccaccaaca cucaacau 19

<210> 314
<211> 19
<212> RNA
<213> Artificial

<220> example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - antisense strand)

<400> 314
auuggcagu guugguagg 19

<210> 315
<211> 19
<212> RNA
<213> Artificial

<220> example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - sense strand)

<400> 315
ucuccaccaac acugacca 19

<210> 316
<211> 19
<212> RNA
<213> Artificial

<220> example ssRNA oligonucleotide (Group A siRNA oligos targeting

"Seite 57"
human cyclophilin B - anti sense strand)

<400>  316
uuggucagug uugguagga

<210>  317
<211>  19
<212>  RNA
<213>  Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - sense strand)

<400>  317
aaacuccuac caacacuga

<210>  318
<211>  19
<212>  RNA
<213>  Artificial

<220>
<223> example SSRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - antisense strand)

<400>  318
ucaguguug uagguuuu

<210>  319
<211>  19
<212>  RNA
<213>  Artificial

<220>
<223> example SSRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - sense strand)

<400>  319
ccaacacuga ccaauaaaa

<210>  320
<211>  19
<212>  RNA
<213>  Artificial

<220>
<223> example SSRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - antisense strand)

<400>  320
uuuuauuggu caguguugg

<210>  321
<211>  19
<212>  RNA
<213>  Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)

<400>  321
caaacuccua ccaacacug

<210> 322
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - anti sense strand)

<400> 322
caguguuggu agaguuug

<210> 323
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)

<400> 323
aacuccuacc aacacugac

<210> 324
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - anti sense strand)

<400> 324
gucaguguug guagguuu

<210> 325
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - sense strand)

<400> 325
aacacugacc auaaaaaa

<210> 326
<211> 19
<212> RNA
<213> Artificial

<220>
<223> example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - anti sense strand)

<400> 326
uuuuuuauug gucaguguu
example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)

caacacugac  caauaaaaa

example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - antisense strand)

uuuuuaugg ucaguguug

gcuacaaaaa cagcaauu

example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)

aaauugcugu uuuguagc

gguuacaaaaa acagcaauu
Artificial example SSRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - anti sense strand)

332
auuucuguu uuuugaccc

333
acacugacca auaaaaaa

334
uuuuuuuuuu ggucagugu

335
uggcuacaaa aacagcaaa

336
uuugcguuu uuguagcca

337
example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin 8 - sense strand)

guaacaaac cuuaccaac

example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - antisense strand)
guugguagga guuuguuac

equivalent ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - sense strand)
cacugaccaau uaaaaaaaa

equivalent ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - antisense strand)
uuuuuuuuu uggucagug

equivalent ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - sense strand)
acugaccaau aaaaaaaaa
uuuuuuuuua uuggucagu

example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)

ggaauuugcu guuuuugua

ggaaauuugcu guuuuugua

cuacaaaaac agcaauuc

cuacaaaaac agcaauuc

gaaauuugcu uuuuuuguag

gaaauuugcu uuuuuuguag

aaaauguggg uuuuuuuuu
example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - antisense strand)

example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)

example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - antisense strand)

example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - sense strand)
example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - sense strand)

```
guuumuuuuuu uuuuuaua
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
aauuuuuuuuuuuuuuuuu
```

```
aaaaaaaa acccacauu
```

```
gguuuuuuuuu uuuuuau
```

```
guuumuuuuuu uuuuuuau
```

```
uuuuuuuuuuu uuuuuuuuu
```

```
```
example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - antisense strand)

358
auuaaaaaaa aaaaaacc 19

359
gguuuuuuu uuuuuuuuu 19

360
uggguuuuu uuuuuuuuu 19

361
uggguuuuuu uuuuuuuuu 19

362
uuaaaaaaaaa aaaaaacc 19

363
example ssRNA oligonucleotide (Group A siRNA oligos targeting human Cyclophilin B - antisense strand)

example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - sense strand)

example ssRNA oligonucleotide (Group A siRNA oligos targeting human cyclophilin B - antisense strand)
example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - sense strand)

aagaucgagg uggagaagc

example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - antisense strand)
gcuucuccac cucgaucuu

example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - sense strand)
agaucgaggu ggagaagcc

example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - antisense strand)
ggcuucuccac ccucgaucuu

example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - sense strand)
ggcgccgcccu caucgcggg
example ssRNA oligonucleotide (Group B siRNA oligos targeting human cyclophilin B - antisense strand)

cccgcgauga
ggcgcggg

19

Example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - sense strand)

ccuucugcgg
cggaugaga

19

Example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - sense strand)

ucucaucgcg
cgcaaggg

19

Example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - antisense strand)

ccgccgcccuc
aucgcgggg

19

Example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - antisense strand)

ccccgcgaug
tagggcggcg

19

Example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - antisense strand)
Artificial example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B – sense strand)

cuuccugcug cugccgga

Artificial example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B – antisense strand)

ucccgccagc agcaggaag

Artificial example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B – sense strand)

gagcgcuuc cccgagaga

Artificial example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B – antisense strand)

ucucaucggg gaaggcgcuc

Artificial example ssRNA oligonucleotide (Group B siRNA oligos targeting human cyclophilin B – sense strand)

gccgccgccc ucaucgcgg
example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - anti-sense strand)

cgcgaugag ggcggccggc

example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - sense strand)
ggcaagaucg agguggaga

example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - anti-sense strand)
ucuccaccuc gaucuugcc

example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - sense strand)
ucuuccgucu gcugccggg

example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - anti-sense strand)
ccgcagcga gcatggaga

example ssRNA oligonucleotide (Group B siRNA oligos targeting human Cyclophilin B - sense strand)
example ssRNA oligonucleotide (Group B siRNA oligos targeting human cyclophilin B - antisense strand)
A. CLASSIFICATION OF SUBJECT MATTER

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

INV. A61K39/39 C12Q1/68

B. FIELDS SEARCHED

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

A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used):

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>HORNUNG VEIT ET AL: <em>Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7</em> NATURE MEDICINE, vol. 11, no. 3, March 2005 (2005-03), pages 263-270, XP002371477 ISSN: 1078-8956 abstract page 264, right-hand column, paragraph 3 - page 265, left-hand column, paragraph 2 page 265, right-hand column, paragraph 1 page 268, left-hand column, paragraph 2</td>
<td>1,3-5, 8-39</td>
</tr>
<tr>
<td>Y</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

X Further documents are listed in the continuation of Box C

X See patent family annex

* Special categories of cited documents

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

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

'L' document which may throw doubts on novelty claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

'S' document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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

'X' document member of the same patent family

Date of the actual completion of the international search: 31 January 2007

Date of mailing of the international search report: 22/02/2007

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

Authorized officer:

Irion, Andrea
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>WO 2005/021044 A (NASTECH PHARMACEUTICAL COMPANY INC; QUAY, STEVEN, C; CUI, KUNYUAN; DAT) 10 March 2005 (2005-03-10) page 5, line 1 - line 7 page 5, line 16 - line 20</td>
<td>9-39</td>
</tr>
<tr>
<td>X</td>
<td>WO 03/086280 A (COLEY PHARMACEUTICAL GMBH; LIPFORD, GRAYSON; BAUER, STEFAN; LIPFORD, G) 23 October 2003 (2003-10-23) the whole document</td>
<td>9-39</td>
</tr>
<tr>
<td>Category</td>
<td>Citation of document, with indication, where appropriate, of the relevant passages</td>
<td>Relevant to claim No.</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
</tbody>
</table>
1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material
   - [X] a sequence listing
   - [ ] table(s) related to the sequence listing

b. format of material
   - [X] on paper
   - [X] in electronic form

c. time of filing/furnishing
   - [X] contained in the international application as filed
   - [X] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purpose of search

2. In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

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

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

2. [X] Claims Nos.: 6, 7 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

   see FURTHER INFORMATION sheet PCT/ISA/210

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

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

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

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

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

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

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.
Continuation of Box II.2

Claims Nos.: 6,7

With respect to claims 6 and 7:
Claims 6 and 7 attempt to obtain protection for a product by defining the product in terms of a screening method used to test its biological activity, i.e. immunostimulatory activity ("Reach-through claims"). Said claims are unclear within the meaning of Article 6 PCT, because in order to determine the scope of the claims the skilled person would have to calculate the IFN-alpha score of all known and yet unknown RNA oligonucleotides and thus are not susceptible of a meaningful search.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CA 2490983 A1</td>
<td>15-01-2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DE 10229872 A1</td>
<td>29-01-2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1521585 A1</td>
<td>13-04-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2005250723 A1</td>
<td>10-11-2005</td>
</tr>
<tr>
<td>WO 2005021044 A</td>
<td>10-03-2005</td>
<td>CA 2536191 A1</td>
<td>10-03-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1664297 A2</td>
<td>07-06-2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2006160123 A1</td>
<td>20-07-2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2480775 A1</td>
<td>23-10-2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1499187 A2</td>
<td>26-01-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2005521749 T</td>
<td>21-07-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2006083184 A</td>
<td>30-03-2006</td>
</tr>
</tbody>
</table>