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(54) **Title:** MODIFICATION OF SMALL RNAS FOR THERAPEUTIC USES

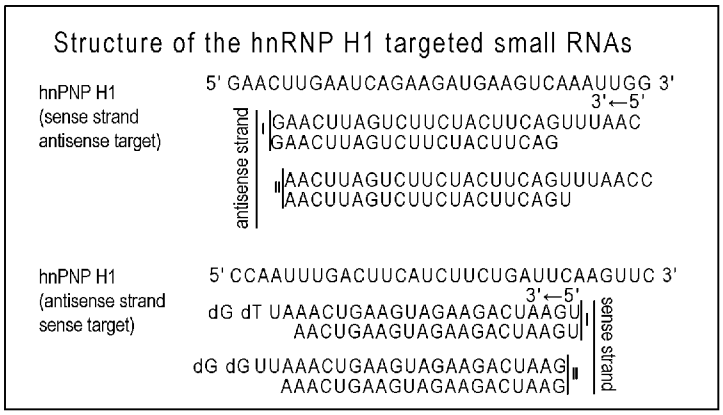

FIG. 1

(57) **Abstract:** Provided are methods for improving stability of small therapeutic RNAs by adding one or more non-templated nucleotides such as cytidines and uridines to the 3' end of the small therapeutic RNAs. Also disclosed are modified small therapeutic RNAs comprising one or more non-templated nucleotides such as cytidines and uridines at the 3' end and pharmaceutical compositions comprising such modified small therapeutic RNAs.

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MODIFICATION OF SMALL RNAS FOR THERAPEUTIC USES

PRIORITY CLAIM

[0001] This application claims priority to U.S. Provisional Application No. 62/690,538, filed on June 27, 2018, which is incorporated by reference herein in its entirety, including drawings.

STATEMENT OF GOVERNMENT INTEREST

[0002] The present invention was made with government support under Grant Nos. R01AI029329 and R01HI074704, awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

[0003] Small RNAs are a type of non-coding RNA having many therapeutic uses due to their specificity, functional diversity and limited toxicity. Many of such small RNAs are in clinical development or available for clinical use. However, small RNAs are unstable in a biological environment and their short *in vivo* half-life due to quick degradation by nucleases limits their therapeutic effects. Therefore, there is a need in the field for modified small RNAs having improved stability and prolonged *in vivo* half-life.

SUMMARY

[0004] In one aspect, provided herein is a method of improving the stability of a small RNA molecule by adding one or more non-templated nucleotides to the 3' end of the small RNA molecule. The method may comprise a step of contacting the small RNA molecule with an exonuclease to remove the 3' overhang of the small RNA molecule before adding one or more non-templated nucleotides to the 3' end of the small RNA molecule. In some embodiments, the exonuclease is 3' to 5' exonuclease. In some embodiments, the exonuclease directly binds to Dicer.

[0005] In some embodiments, the small RNA molecule is cytidylated by adding one or more cytidines to the 3' end of the small RNA molecule. For example, one cytidine, two cytidines, three cytidines, four cytidines, or five cytidines are added to the 3' end of the small RNA molecule. In certain embodiments, adding one or two cytidines to the 3' end of the small RNA molecule improved the stability of the small RNA molecule without

any undesired impact on the therapeutic effects of the small RNA molecule. In some embodiments, the small RNA molecule is uridylated by adding one or more uridines to the 3' end of the small RNA molecule. For example, one uridine, two uridines, three uridines, four uridines, or five uridines are added to the 3' end of the small RNA molecule. In certain embodiments, adding one or two uridines to the 3' end of the small RNA molecule improved the stability of the small RNA molecule without any undesired impact on the therapeutic effects of the small RNA molecule. In some embodiments, the *in vivo* stability of the small RNA molecule is improved by the modifications disclosed above. The improved *in vivo* stability is demonstrated by prolonged half-life and decreased degradation of the modified small RNA molecule in cells in comparison to the corresponding unmodified small RNA molecule in cells.

[0006] In some embodiments, the small RNA molecules encompassed in this disclosure include but are not limited to small RNAs that are capable of inducing RNA interference (RNAi), such as small interfering RNA (siRNA), short hairpin RNA (shRNA), microRNA (miRNA) and Piwi-interacting RNAs (piRNAs). In some embodiments, the small RNA molecules include small activating RNAs (saRNAs) which are small double-stranded RNAs (dsRNAs) capable of inducing transcriptional gene activation. In some embodiments, the small RNA molecules disclosed herein are Dicer-derived, e.g., produced by cleaving double-stranded RNAs with Dicer. In some embodiments, the small RNA molecules disclosed herein bind to the Argonaute protein to form a RNA-induced silencing complex (RISC). In some embodiments, the small RNA molecules disclosed herein have a size of less than 50 base pairs (bps), less than 45 bps, less than 40 bps, less than 35 bps, less than 30 bps, less than 35 bps, less than 30 bps, less than 25 bps, less than 20 bps, or less than 15 bps. In some embodiments, the small RNA molecules disclosed herein have a size of about 15 bps, about 16 bps, about 17 bps, about 18 bps, about 19 bps, about 20 bps, about 21 bps, about 22 bps, about 23 bps, about 24 bps, about 25 bps, about 26 bps, about 27 bps, about 28 bps, about 29 bps, or about 30 bps.

[0007] In another aspect, disclosed herein is a modified small RNA molecule having an improved stability comparing to the unmodified small RNA molecule. Preferably the *in vivo* stability of the small RNA molecule is improved. The modified small RNA molecule comprises the entire nucleotide sequence of the corresponding unmodified

small RNA molecule, and one or more non-templated cytidines added to the 3' end of the unmodified small RNA molecule. For example, one cytidine, two cytidines, three cytidines, four cytidines, or five cytidines are added to the 3' end of the small RNA molecule. In certain embodiments, adding one or two cytidines to the 3' end of the small RNA molecule improved the *in vivo* stability of the small RNA molecule without any undesired impact on the therapeutic effects of the small RNA molecule. Alternatively, the modified small RNA molecule comprises the entire nucleotide sequence of the corresponding unmodified small RNA molecule, and one or more uridines added to the 3' end of the unmodified small RNA molecule. For example, one uridine, two uridines, three uridines, four uridines, or five uridines are added to the 3' end of the small RNA molecule. In certain embodiments, adding one or two uridines to the 3' end of the small RNA molecule improved the *in vivo* stability of the small RNA molecule without any undesired impact on the therapeutic effects of the small RNA molecule.

[0008] In another aspect, disclosed herein is a pharmaceutical composition comprising a therapeutically effective amount of a modified small RNA molecule having an improved stability comparing to the unmodified small RNA molecule. The modified small RNA molecule comprises the nucleotide sequence of the unmodified small RNA molecule, and one or more cytidines added to the 3' end of the unmodified small RNA molecule. For example, one cytidine, two cytidines, three cytidines, four cytidines, or five cytidines are added to the 3' end of the small RNA molecule. In certain embodiments, adding one or two cytidines to the 3' end of the small RNA molecule improved the *in vivo* stability of the small RNA molecule without any undesired impact on the therapeutic effects of the small RNA molecule. Alternatively, the modified small RNA molecule comprises the nucleotide sequence of the unmodified small RNA molecule, and one or more uridines added to the 3' end of the unmodified small RNA molecule. For example, one uridine, two uridines, three uridines, four uridines, or five uridines are added to the 3' end of the small RNA molecule. In certain embodiments, adding one or two uridines to the 3' end of the small RNA molecule improved the *in vivo* stability of the small RNA molecule without any undesired impact on the therapeutic effects of the small RNA molecule. In some embodiments, the pharmaceutical composition further comprises one or more pharmaceutically acceptable carriers or excipients.

[0009] In another aspect, disclosed herein is a method of treating various diseases and conditions treatable by small therapeutic RNAs in a subject. Such diseases and conditions include but are not limited to cancer, physiological and metabolic disorders, cardiovascular diseases, monogenic diseases, neurological diseases, ocular diseases, and viral infections. Various diseases are treatable by small therapeutic RNAs, and can benefit from the technology disclosed herein. For a given disease or condition, a particular small therapeutic RNA for treating the disease or condition can be stabilized by modification, thereby enhancing its therapeutic effects. The method comprises administering to a subject suffering from a disease or condition a therapeutically effective amount of the modified small RNA molecule or the pharmaceutical composition comprising the modified small RNA molecule, both described above. In some embodiments, the subject suffers from cancer such as colon cancer. In some embodiments, the small RNA molecule targets hnRNP H1.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] This application contains at least one drawing executed in color. Copies of this application with color drawing(s) will be provided by the Office upon request and payment of the necessary fees.

[0011] Figure 1 illustrates the structure of certain small RNAs used in this disclosure, such as hnRNP H1 targeted small RNAs.

[0012] Figure 2 demonstrates the gene knockdown efficiency of the Dicer substrate siRNAs (DsiRNAs) and siRNAs. HCT116 cells were co-transfected with psi-hnRNPH1_AS or _S reporter plasmid and a small RNA (3 pM to 50 nM). Cell lysates were collected 24-hours post-transfection and used in the Dual-Luciferase Reporter assay system.

[0013] Figure 3 shows the percentage of antisense (bottom bars) and sense strands (top bars) in total RNA, Dicer immunoprecipitation and Ago2 immunoprecipitation.

[0014] Figure 4 demonstrates the strand stability of the sense and antisense RNAs. The count number of each small RNA in the cell is determined by deep-sequencing. The cells were transfected with small RNAs such as DSiRNAs and siRNAs and after two days, the total RNA was recovered for sequencing.

[0015] Figure 5 compares the efficiency and stability of the DsiRNAs and siRNAs. The efficiency was tested by luciferase assay for siRNA gene knockdown activity. The stability was determined by the normalized count numbers of each siRNA strand by deep-sequencing. S represents the knockdown activity or count numbers of the sense strand from siRNAs. AS represents the knockdown activity or count numbers of the anti-sense strand from siRNAs.

[0016] Figure 6 compares the efficiency and stability of the antisense strand in DsiRNAs.

[0017] Figures 7A-7B show the process (Figure 7A) and results (Figure 7B) of immunoprecipitation for Dicer and Ago2. HCT116 cells were co-transfected with the small RNAs and FLAG-DICER or FLAG-AGO2 plasmid and then Dicer and Ago2 were immunoprecipitated using anti-FLAG beads and eluted from the bead using FLAG peptide. Cell extracts and FLAG-eluted proteins (FLAG-IP) were separated by SDS-PAGE, stained with silver staining solution.

[0018] Figure 8 shows the normalized count number of small RNAs from the total RNA, small RNAs bound to Dicer, and small RNAs bound to Ago2.

[0019] Figure 9 shows the top 10 reads for deep sequencing of perfectly aligned small RNAs from corrected total RNA, Ago2 and DICER immunoprecipitation (IP). To calculate the normalized count numbers, reads for each strand were divided by the total number of each deep-sequencing.

[0020] Figure 10 shows the percentage of uridylation of the small RNAs in HCT116 cells. Gray bars represent no addition in non-selected strand, black bars represent no addition in selected strand, light green bars represent uridine addition in non-selected strand, and dark green bars represent uridine addition in selected strand. Non-templated uridine was added at the 3' end of the small RNAs and the non-modified and modified small RNAs were used to transfect HCT116 cells. AS top bars: U addition in non-selected strand; AS bottom bars: no addition in non-selected strand; S top bars: U addition in selected strand; S bottom bars: no addition in selected strand.

[0021] Figure 11 is a histogram of uridylation demonstrating that the mono-uridylated small RNAs are favorable to Dicer. In the right panel, left bars are no treatment, middle bars are Dicer IP, and right bars are Ago2 IP.

[0022] Figure 12 shows that the mono-uridylation predominantly exhibited in the sequencing cohort of Dicer bound siRNAs.

[0023] Figure 13 shows the five most representative sequence variants of uridylated siRNAs in different condition. The black bar shows nucleotide matched small RNAs. The green bar represents nucleotide of U. The percentage shows expression level of each sequence. The graphs on the right show that the proportion of uridylation small RNAs in total RNA, Ago2 IP and Dicer IP.

[0024] Figures 14A-14D show that mono-uridylated siRNA affected by Dicer but not Ago2. Figure 14A shows the efficiency of siRNA (middle bar) and uridylated siRNA (right bar) in HCT116 (no treatment, left bar), Dicer knockdown HCT116 (siDicer), and Ago2 deficient HCT116 (siAgo2). siRNA H1 and H2 targeted hnRNPH1 was designed (Figure 14B) and siRNA efficiency was tested in HCT116, Dicer deficient HCT116, and Ago2 knockdown HCT116 (Figures 14C-14D). In Figure 14B, the top strands are antisense strands for siRNA H1 and siRNA H2. Figure 14C shows the siRNA efficiency of antisense (left bar) or sense strand (right bar). Both siRNAs were selected for the sense strand. In Figure 14D, for each group of siRNA H1 and siRNA H2, left bar is siRNA, and right bar is uridylated siRNA.

[0025] Figures 15A-15D show that the normalized count number of miRNA reduced in Dicer immunoprecipitation (DIP) but uridylated miRNA increased in DIP. Figures 15A-15B show the normalized count number of individual miRNA in total RNA (-Con.) and RNA from Dicer immunoprecipitation (DIP). The mean of the normalized count number reduced in DIP (Figure 15B). Error bar represents SEM. Figures 15C-15D show the percentage of uridylated sequence in individual miRNA. Each dot represents an individual miRNA. The percentage of uridylated sequence of miRNA reduced in DIP.

[0026] Figures 16A-16B show the knockdown efficiency of siRNA targeted Dicer (siDICER), Ago2 (siAGO2) (Figure 16A) or Eri1 (siEXOD) (Figure 16B). siRNA targeted Dicer, Ago2 and Eri1 were transfected and expression levels of Dicer, Ago2 and Eri1 were determined by RT-qPCR. These results show the knockdown of dicer, Ago2 and Eri1 by each siRNA.

[0027] Figures 17A-17C show the normalized count number of miRNAs or siRNAs. Figures 17D-17E show the population of uridylated miRNA or siRNAs. The fold change

was calculated by dividing the normalized count number in Eri knockdown cell by the normalized count number of the control from total RNA.

[0028] Figure 18A shows that miRNA expression measured by deep-sequencing in Eri1 knockdown HCT116 cell line. Scatter plot matrix of each miRNA array for the Log2 fold change (x-axis) and normalized counts (y-axis) of miRNA in Eri deficient cells. The relative miRNA expression levels from treated Eri-knockdown cells to miRNA independent of their total expression levels was examined. Figures 18B and 18C show the uridylation percentage of each miRNA. Error bar represents SEM for relocates (n=4) in Figure 18C. Figure18D shows that uridylation population of individual miRNA to grouped up-regulation (>1, more than 2 folds, right pink bar) or down-regulation (<1, less than 2 folds, left blue bar) in Eri-deleted cells depending on Figure 18A. Figure 18E shows the mean of percentage of uridylated miRNA in up or down grouped miRNA depending on Eri1. Figures 18F-18G show the grouped miRNA depend on Dicer.

[0029] Figure 19 shows the siRNA efficiency over the period of one week.

[0030] Figure 20 shows immunoprecipitation for Dicer of Ago2 in Eri knockdown HCT116 (left gel picture: silver stain). The graph on the right shows the normalized count number of transfected siRNAs (siRNAs) and endogenous miRNAs (miRNAs) in HCT116 (-: black bar) and Eri deficient HCT116 (siEXOD: grey bar) by deep-sequencing.

[0031] Figure 21 shows the different uridylation percentage between siRNAs and miRNAs. The graph on the left shows that addition percentage included with A, C, U, and G nucleotide in the 3' end (upper panel) and U addition percentage of siRNA (bottom panel) in HCT116 (black bar) and Eri knockdown HCT116 (grey bar). The graph on the right shows the percentage of A, C, U and G added miRNA (upper panel) and the percentage of U added miRNA (bottom panel) in HCT116 (black) and Eri1 deficient cells (grey). Dots show the mean value. Uridylated siRNA reduced in Eri1 knockdown HCT116, but uridylated miRNA increased in same condition.

[0032] Figure 22 shows the mechanism of mono-uridylated siRNA.

[0033] Figures 23A-23B show non-templated addition at the 3' end of transfected small RNA (Figure 23A) and small RNAs bound to Dicer (Figure 23B). The non-templates C addition dramatically increased in cohort of Dicer immunoprecipitation.

[0034] Figure 24 compares C addition and U addition of the Dicer and Ago2 binding

pattern of 3' end non-templated smRNAs. Left bar: cell lysates from total RNA, middle bar: cell lysates from collected by Dicer, and right bar: cell lysates from immunoprecipitated Ago2.

[0035] Figure 25 shows the binding efficiency of small RNAs. EMSA was performed with or without extract of HCT116 cells and a FAM-labelled oligonucleotide. C: single C addition, CC or 2C: double C addition, U: single U addition, 5U: five U addition.

[0036] Figure 26 shows the EMSA results of siRNA, siRNA with single C addition and siRNA with double C addition in protein with immunoprecipitated Dicer from Lysates of dicer overexpressed or knockdown HCT116 cells. The binding efficiency did not change comparing the siRNA with Dicer and without Dicer, but C added and CC added siRNAs reduced the binding efficiency without Dicer. The sequences (5'→3') are as follows: siRNA S: UGAAUCAGAAGAUGAAGUCAA, siRNA AS: GACUUCAUCUUCUGAUUCAAG, siRNA_C S: UGAAUCAGAAGAUGAAGUCAAC, siRNA_C AS: GACUUCAUCUUCUGAUUCAAG, siRNA_CC S: UGAAUCAGAAGAUGAAGUCAACC, and siRNA_CC AS: GACUUCAUCUUCUGAUUCAAG.

[0037] Figure 27A shows co-localization of siRNAs and CTP, Figure 27B shows the localization of Dicer by Z-stack and Dicer existed in nucleus. Purple stain is Dicer antibody, blue stain is DAPI. Figure 27C shows the localization of C added small RNA. The labeled siRNA and CTP locate in nucleus and cytoplasm. The figures show that C added siRNA with dicer can move between nucleus and cytoplasm.

[0038] Figure 28 shows the various locations of non-template addition in the 3' end of siRNAs. The green peak is the position of U addition in siRNA 3' end, yellow peak is the C addition. The upper panel is the position of U or C addition from total cell Lysates, the bottom panel is from Dicer immunoprecipitation. There is one nucleotide difference in the position of U and C addition.

[0039] Figure 29 compares the effects of trimming vs. of the number of addition of C or U.

[0040] Figure 30 compares the 3' end nucleotides of transfected small RNAs from total RNA lysate and siEri1treated cell. The position of siRNA 3' end goes to higher number of nucleotide in Eri1 deficient cells (shown by arrows). Eri1 is a 3' to 5'

exonuclease.

[0041] Figure 31 shows the effects of sense and antisense strands of the smRNAs in –control (HCT116) and Eri1 knockdown HCT116. Eri1 did not affect siRNA efficiency.

[0042] Figure 32 shows *in vitro* cytidylation of siRNA in Dicer with nucleotidyltransferase. Immunopurified TRNT1, MTPAP, ZCCH11(TUT4) and ZCCH6 (TUT4) was incubated with the labeled siRNA (AS: cy3, S: cy5) and CTP. These proteins can add C nucleotide in siRNA with assistance of Dicer.

[0043] Figure 33 shows the results of *in vitro* C addition assay. siRNA: Cy5.5 labeled SI_S of 5' end; -: without protein; T: with TRNT1 protein from IP; Marker(M): 21nt: Cy5.5 labeled SI_S of 5' end, 22nt: Cy5.5 labeled SI_S_C of 5' end, 23nt: Cy5.5 labeled SI_S_CC of 5' end. SiRNA added the CC addition with TRNT1 protein.

[0044] Figure 34 shows the efficiency of small RNAs with non-template addition of C. Single cytidylated and double cytidylated siRNAs did not affect siRNA efficiency. The siRNAs were inhibited more by the siDicer than siAGO2. Left: no treatment, middle: siDicer, and right: siAgo2.

[0045] Figure 35 shows the efficiency of small RNAs with non-template addition of C in HCT116 cells. The siRNA efficiency of CC added siRNA reduced in Dicer deficient HCT116 (HCT116 DCR-/-: the exon 5 of Dicer gene was deleted) and in double knockdown HCT116 which transfected siDicer in HCT116 DCR-/-. The graph on the right shows western blot results of Dicer and GAPDH. Dicer knockdown in HCT116 DCR -/-.

[0046] Figure 36 shows the efficiency of small RNAs with non-template addition of C by the hnRNPH1 expression level. CC added siRNA can inhibit endogenous gene of hnRNPH1 and shows better efficiency than siRNA.

[0047] Figures 37A and 37B show the results of small RNA degradation assay in cytoplasm and nucleus. The 5'→3' sequences of siRNA: UGAAUCAGAAGAUGAAGUCAA, siRNA_C: UGAAUCAGAAGAUGAAGUCAAC, and siRNA_CC: UGAAUCAGAAGAUGAAGUCAACC. XRN is a 5' to 3' exonuclease. The siRNA (black dot), C-added siRNA (green square), and CC-added siRNA (purple triangle) reacted with cell lysate from fraction of cytoplasm or nucleus (upper panel), degraded siRNA in SDS-PAGE gel was detected by typhoon scanner. The degradation percentage calculated the band intensity of degraded siRNAs. CC-added siRNA delays degradation

in nucleus lysate. This result disappears in nucleus lysate with siXRN which knockdown XRN protein [down graph].

[0048] Figure 38 shows protein knockdown of XRN2 by Western blot. XRN2 was expressed in nucleus. The expression level of XRN2 reduced 60% or 43% in HCT116 XRN2-/- with transfected siXRN2. Tubulin was the marker of cytoplasmic Lysates.

[0049] Figure 39 shows the efficiency of siRNAs by luciferase assay. The efficiency of siRNA reduced in XRN2 knockdown HCT116 (XRN-). Left bars: HCT116; right bars: XRN-.

[0050] Figure 40 shows the miRNA expression level from Dicer immunoprecipitation, total RNA or Ago1 immunoprecipitation. T: total lysate; N: the fraction of nucleus lysate; C: the fraction of cytoplasm lysate.

[0051] Figure 41 shows the expression levels of individual miRNAs in Dicer IP (left) and Ago2 IP (right). Dicer favored miRNAs bound with Dicer in Dicer immunoprecipitation were collected (marked by red dots), which distributed mostly on the right in Dicer IP but more evenly distributed in Ago2 IP. The patterns of individual miRNA expression were different in Dicer IP and Ago2 IP.

[0052] Figure 42 shows the expression levels of individual miRNAs in Ago1, Ago3, and Ago4 IP.

[0053] Figure 43 shows the expression levels of individual miRNAs in Dicer or Ago2 IP. Dark blue shows the selected strand of miRNA. Light blue shows the non-selected strand of miRNA.

[0054] Figure 44 shows cytidylated miRNA in fraction of nucleus and cytoplasm bound with Dicer. The results show that cytidylated miRNA bound with Dicer.

[0055] Figure 45 shows the expression levels of miR-30d, miR-30e and miR31. The miR30d and miR30e were down-regulated in Dicer immunoprecipitation from total RNA (Total), fraction RNA of nucleus (Nu) and cytoplasm (Cy). However, miR31 expression levels in all Dicer immunoprecipitation were increased.

[0056] Figure 46 shows degradation rate of miRNAs and cytidylated miRNAs in fraction of cytoplasm (Cy), nucleus (Nu), and – control (-). miRNAs and cytidylated miRNAs with buffer (-), lysates from cytoplasm (Cy) and nucleus (Nu) were reacted and remaining miRNAs after reaction were detected in SDS-PAGE gel. The has-miR30d and

has-miR-30e samples which did not bind with Dicer showed same degradation ratio. However, has-miR31 bound with Dicer (dicer favor miRNA) increased the band intensity in reaction with cytidylated miR31.

[0057] Figure 47 shows the long-term stability of 3' C added siRNAs.

[0058] Figure 48 shows the long-term stability of 3' C added siRNAs. This data shows that cytidylated siRNA targeted cancer related gene of Vim in controlling the stability of siRNA.

[0059] Figure 49 shows the efficiency of small activate RNA (saRNA) by RT-qPCR. CEBPa targeted saRNA which can activate the expression of CEBPa gene and designed CC-added saRNA (middle bar) and U-added saRNA (right bar). The CC-added saRNA and U-added saRNA improved the efficiency of saRNA.

[0060] Figure 50 shows the effects of siRNAs on tumor reduction.

DETAILED DESCRIPTION

[0061] Methods of improving the stability of small therapeutic RNAs are provided herein. The method includes adding one or more non-templated nucleotides, such as cytidines and uridines, to the 3' end of the small therapeutic RNA molecule. Such modified small RNAs can stably bind to Dicer and escape the cell defense system to stably exist in cells for a longer time such that the modified small RNAs can achieve their therapeutic effects. Various small therapeutic RNAs are encompassed by this disclosure. In some embodiments, the small RNAs include, for example, siRNA, shRNA, miRNA, and saRNA. In some embodiments, the small RNAs include Dicer substrate small RNAs, such as Dicer substrate siRNAs (DsiRNAs), which can bind to Dicer and result in increased RNAi activities.

[0062] Adding non-templated nucleotides to the 3' ends of small RNA molecules can have a profound impact on the stability and biological function of the small RNA molecules. Evidence accumulated over the past few decades has established roles for uridylation and adenylation in small RNA stabilization and degradation. 3'-end formation of small RNAs occurs through a delicate balance between the removal and the addition of nucleotides. By sequencing transfected small RNAs of total RNA and associated with Dicer-containing ribonucleoprotein (RNP), the majority of non-template mono-uridine in

3' end of small RNAs were demonstrated to be associated with Dicer.

[0063] miRNAs and siRNAs appeared to be distinguished in two primary ways. First, miRNAs were viewed as endogenous and purposefully expressed products of an organism's own genome, whereas siRNAs were thought to be primarily exogenous in origin, derived directly from the virus, transposon, or transgene trigger. Second, miRNAs appeared to be processed from stem-loop precursors with incomplete double-stranded character, whereas siRNAs were found to be excised from long, fully complementary double-stranded RNAs (dsRNAs). Despite these differences, the size similarities and sequence-specific inhibitory functions of miRNAs and siRNAs immediately suggested relatedness in biogenesis and mechanism. As demonstrated herein, EXOD (ERI protein) enriched the siRNA stability. EXOD stimulates the long-term stability of mono-uridylated siRNAs in colon cancer cells. This feature makes mono-uridylated siRNA a more powerful therapeutic agent.

[0064] As demonstrated herein, non-template uridine addition was predominant in Dicer immunoprecipitation with both the selected strand and non-selected strand of the small RNAs. The experimental data shows that the mono-uridylated small RNAs are favorable to Dicer. However, EXOD enriched siRNA stability but not miRNA. Mono-uridine modified siRNA shows the efficiency for a longer term in colorectal cancer, and therefore such modified siRNAs have therapeutic uses in treating cancer.

[0065] As disclosed herein are cytidylated small RNAs which demonstrated improved stability, thereby enhancing the therapeutic effects comparing to unmodified small RNAs. It was demonstrated that small RNAs such as siRNA and CTP were co-localized in the nucleus of the cell.

[0066] For exogenous small RNAs such as siRNAs, the small RNA molecules are modified based on the known sequences of such RNA molecules by adding one or more non-templated nucleotide to the 3' end of the known sequences. Optionally, the small RNA molecules are treated with an exonuclease to remove the 3' overhang before cytidylation.

[0067] For endogenous small RNAs such as miRNAs, immunoprecipitation of Dicer or Argonaute complex are performed to recover endogenous small RNAs captured by Dicer or Argonaute. Sequence analysis is performed to determine the sequence of

the captured endogenous small RNAs. Based on the obtained sequence, modified small RNAs are produced by adding one or more non-templated cytidines or uridines to the 3' end of the sequence. Such modified miRNAs are transfected to cells for therapeutic uses.

[0068] The following examples are intended to illustrate various embodiments of the invention. As such, the specific embodiments discussed are not to be construed as limitations on the scope of the invention. It will be apparent to one skilled in the art that various equivalents, changes, and modifications may be made without departing from the scope of invention, and it is understood that such equivalent embodiments are to be included herein. Further, all references cited in the disclosure are hereby incorporated by reference in their entirety, as if fully set forth herein.

EXAMPLES

Example 1: Materials and Methods

[0069] **Determination of IC50 value**: Generation of the psiCHECK-hnRNPH_AS (antisense reporter) and psiCHECK-hnRNPH_S (sense reporter) vectors used in this study was previously disclosed (Sakurai et al., 2011). HCT116 cells were co-transfected in a 48-well format (50,000 cells/well) with psiCHECK-hnRNPH-AS or psiCHECK-hnRNPH-S vector (50 ng), 3 pM – 50 nM small RNAs and 1 μL Lipofectamine 2000 (Invitrogen) per well. Cells were lysed in 1X Passive Lysis Buffer (Promega) 24 hours after transfection and the efficiency and duration of small RNA mediated gene silencing analyzed using the Dual-Luciferase Reporter System (Promega) and a Veritas microplate luminometer (Turner Biosystems). The average was calculated from the replicates to set Renilla/Firefly luciferase expression to 100%. An IC50 curve was generated using Prism 5.01 software (GraphPad). Sigmoidal dose response was calculated according to γ = Bottom + (Top−Bottom)/(1 + 10) λ ((LogEC50-χ)); where χ is the logarithm of concentration and γ is the response.

[0070] For siDicer or siAGO2 treatment, HCT116 cells were seeded in a 24-well plate (100,000 cells/well) and transfected with 20 nM siRNA-targeting Dicer or AGO2. The sequences of the siRNAs used in this study were: Dicer, 5'-UUUGUUGCGAGGCUGAUUCdTdT-3'; AGO2, 5'-GCACGGAAGUCCAUCUGAAdTdT-3'. Twenty-four hours later, the siRNA-treated cells were lysed and used for Dual Luciferase assays and RT-qPCR, respectively. The same procedure described above

was used for Dual Luciferase assays.

[0071] **Flag-tag immunoprecipitation**: HCT116 cell cultures (~80% confluent) in 10-cm dishes were co-transfected with a Flag-tagged Argonaute or Dicer expression plasmid and each small RNA and incubated for 48 hours. Cells were then washed with ice-cold 1×PBS, followed by incubation (15 minutes) in 1 mL of lysis buffer (10 mM Tris–HCl pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 5 mM DTT, 2 M NaCl and 1 X Complete EDTA-free protease inhibitor cocktail; Roche). Cells were then scraped off the plate and the suspensions were supplemented with recombinant RNasin (final concentration 0.4 U/μL, Promega). The resulting mixtures were centrifuged for 20 minutes at 13,000 rpm. The supernatant was recovered and centrifuged for 5 minutes at 13,000 rpm. Anti-Flag agarose beads (40 μL; Sigma) were pre-blocked for 2 hours at 4 °C in W1 buffer (0.5% Nonidet P-40, 150 mM NaCl, 2 mM MgCl2, 2 mM CaCl2, 20 mM Tris–HCl, pH 7.5, 5 mM DTT and 1X Complete EDTA-free protease inhibitor cocktail) containing 1 mg/mL yeast tRNA and 1 mg/mL BSA. Anti-Flag M2 affinity beads (Sigma) were mixed with the supernatant protein extract according to the manufacturer's recommendations and then incubated (3 hours, 4°C). The gel was washed with the same buffer, and the beads were resuspended in 3X Flag peptide for elution and incubated (30 minutes, 4°C). RNAs were eluted from the beads by phenol extraction.

[0072] **Small RNA deep sequencing**: HCT116 cells were split in 10-cm dish to 70–80% confluency in DMEM media one day prior to transfection. Cells were transfected with the small RNAs (50 nM) using lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Forty-eight hours after transfection, total RNA was isolated with TriZol reagent (Invitrogen, Carlsbad, CA) for Illumina deep sequencing.

[0073] **Bioinformatic analysis**: To identify the most frequent sense and anti-sense products from each dsiRNA molecule, Novoalign v2.05 (www.novocraft.com) was used to align the sequences generated from Illumina Pipeline v1.6 to the sense and antisense strand of each siRNA molecule. All subsequent analysis was done using the R statistical environment and Bioconductor packages "Biostrings" and "ShortRead" (Morgan et al., 2009). Only sequences that could be aligned to the siRNA sequences without mismatches were retained. The relative start and end position of identified sequences on the siRNA sequence were summarized based on their aligned position and

length. The frequency for each product was counted.

[0074] To examine if nucleotides were added at either end of the dicer-processed product, the raw sequences were matched to the siRNA anti-sense sequence with a seed size of 16 after removing the 3'-adapter with Bioconductor package "ShortRead". For example, for an siRNA sequence length of 23, the Illumina sequences were aligned to 8 seeds, which included the sub-sequence from bases 1–16, 2–17, and so on, of the original siRNA sequence. The matched sequences were then reduced to a set of unique sequences along with the frequencies of occurrence. This set of sequences was then aligned along with the siRNA reference sequence by using the ClustalX2 multiple alignment tool (Larkin et al., 2007) and not allowing gaps. The multiple aligned sequences were visualized and exported with JalView (Waterhouse et al., 2009). Extra bases at either end of the product were highlighted manually.

[0075] **Confocal Microscopy**: A two-Photon Zeiss LSM510 META Inverted microscope (Carl Zeiss, Jena, Germany) was used. Images were taken with the 40X or 63X water immersion C-APOCHROMAT objective lenses (N.A. = 1.2) using multi-track configuration. The following filter sets were used: HFT UV488/543/633 with DBS NFT 490 or NFT 545, HFT KP 650 or mirror or none. An argon laser (488 nm) was used to excite Alexa 488 and emission was collected using a 500-550 nm band pass filter. A helium-neon laser (543 nm) was used to excite Alexa 555 and emission was collected using a 565–615 nm band pass filter. A Ti-Sapphire (Coherent, Inc) laser (790 nm) was used to excite DAPI and emission was collected using a 435-485 nm band pass filter.

[0076] **Statistical analysis**: All data represent the mean±S.D. Student's t-tests were performed using GraphPad Prism v4 (GraphPad Software).

Example 2: Difference between miRNA-mediated gene silencing and RNA stability

[0077] The efficacy of siRNAs targeting the hnRNP H1 mRNA was examined using the sequences shown in Figure 1. Two versions of the small RNAs (I and II), which differed by only a single base pair, were designed. To address which features of siRNAs determine gene knockdown and activity strand selectivity, dual luciferase reporter assays were performed, in which the target hnRNP H1 sequence was cloned into the psiCHECK2 vector in either the sense or antisense orientation within the 3' untranslated region (UTR) of the Renilla luciferase gene. For this translation-based assay, the interference effects

correlate well with small RNA efficiency at inhibiting mRNA translation of the Renilla luciferase gene. The relative silencing efficiencies of the sense versus antisense reporters were determined by which strand was most efficiently incorporated into RISC and advanced to the miRNA-mediated gene silencing pathway. Dose-dependent inhibition assays were performed to determine the IC50 values (i.e., the concentration of the small RNA for which its efficiency of gene silencing was reduced by half) for both strands of the small RNAs (Table 1, Figure 2).

Table 1. Determination of hnRNPH1 small RNAs IC_{50} value

IC50 [pM]	siRNA I	siRNA II
Antisense strand (targeted sense strand)	89.92 ± 1.206	43.55 ± 1.181
Sense strand (targeted antisense strand)	4.278 ± 1.213	77.41 ± 1.228
AS vs S*	0.05	1.78
S vs AS**	21	0.56

* AS vs S (S preference): IC_{50} value of antisense strand/IC_{50} value of sense strand;

** S vs AS (AS preference): IC_{50} value of sense strand/IC_{50} value of antisense strand.

[0078] siRNAs targeting hnRNP H1 were tested at concentrations ranging from 0.001 to 50 nM. Asymmetric knockdown of the hnRNP H1 targets by the antisense and sense strands of both types of small RNAs was observed; the antisense strand of small RNAs targeted the sense mRNA strand of hnRNP H1. However, strand bias differed significantly between the siRNA I and II for a given version of these small RNAs. The sense strand of siRNA I (SI) had a 21-fold difference in IC50 value in preference of targeting the antisense mRNA strand of hnRNP H1, while the preference of siRNA II (SII) for targeting the sense mRNA strand of hnRNP H1 was only slightly greater than unity (Table 1 and Figure 2). One of the two strands was preferred to serve as the guide of the sense strand of siRNA I and antisense strand of siRNA II. Thus, both the sequence targeted and the form (I versus II) of the small RNA influenced strand selectivity.

[0079] To identify the sequences that interact with AGO2 and DICER, FLAG-tagged AGO2 and DICER were expressed in HCT116 cells by transfection, and cell lysates were subjected to affinity purification with an anti-FLAG bead. Protein and RNA complexes eluted with 3X FLAG peptide, and the purified RNA was identified using deep-

sequencing (Figure 7A).

[0080] To further investigate the strand selectivity of the four small RNAs tested, an Illumina GAII sequencer was used to deep-sequence a transfected short RNA library from HCT116 cells. The extracted tags were then mapped to individual small RNAs. In this experiment, the number of reads obtained reflects the relative existence and stability of a given strand species (Table 2).

Table 2. Transfected small RNAs counts number of total RNA, Dicer RNA and Ago2 immunoprecipitation

	siRNA I			siRNA II		
	Total RNA	**Dicer IP**	**AGO2 IP**	**Total RNA**	**Dicer IP**	**AGO2 IP**
Total Reads	21,388,815	10,606,494	22,003,133	25,345,783	1,940,450	16,282,902
Matched to AS	45,542	73,135	1,158,830	405,642	146,281	555,069
Matched to S	2,853,298	651,505	422,828	419,135	19,040	425,620
Mapped Total	2,898,840	724,640	1,581,658	824,777	165,321	980,689
% of AS[1]	0%	1%	5%	2%	8%	3%
% of S[2]	13%	6%	2%	2%	1%	3%
% mapped to smRNA[3]	14%	7%	7%	3%	9%	6%
AS vs S[4]	0.0	0.1	2.7	1.0	7.7	1.3
S vs AS[5]	62.7	8.9	0.4	1.0	0.1	0.8

1. % of AS: matched to AS X 100/total reads
2. % of S: matched to S X 100/total reads
3. mapped to smRNA%: mapped total X 100/total reads
4. AS vs S (AS preference): % of AS/% of S
5. S vs AS (S preference): % of S/% of AS

[0081] To compare the levels of each small RNA in the different samples, lists of the most prevalent species of short RNAs derived from both strands of transfected species were generated. The sequences, normalized counts and percent abundance of transfected small RNAs are shown in Table 2. The mapped total of siRNA I_sense which was the best gene knockdown efficiency in Table 1, had the highest normalized count number of 2,853,298 from lysates of total RNA.

[0082] To compare the antisense and sense strands of transfected small RNAs, the percentage of antisense or sense strands was calculated (Figure 3). The antisense strand is the non-selective strand in the version I of the small RNA. The antisense strand of siRNA I immediately degraded in cells, this means that the antisense strand of siRNA I is the passenger strand, and the other strand of the selected strand of sense appeared to be the high-efficiency strand.

[0083] To understand the uridylation in siRNAs, the percentage of uridylation in each strand of siRNAs from total, Ago2 bound and Dicer bound RNA pools was analyzed (Figure 10). Interestingly, the percentage of uridylation increased in DICER bound small RNA pools. However, Ago2 bound siRNA showed a percentage similar to total RNA. These results suggest that uridylated siRNA can bind to Dicer but not Ago2.

Example 3: Pattern of uridylation

[0084] To investigate the association between the extent of U addition and truncation in 3' end of siRNAs, the siRNA sequences from small RNA deep sequencing data were analyzed (Tables 3-5).

Table 3. U addition from total lysate

	Total Count				Percentage			
	siRNA I		siRNA II		siRNA I		siRNA II	
Number of U addition	GS	PS	GS	PS	GS	PS	GS	PS
0	37,694	1,821,412	279,231	388,781	85.3	64.0	69.2	93.2
1	4,516	315,505	27,282	5,561	10.2	11.1	6.8	1.3
2	371	135,974	7,441	954	0.8	4.8	1.8	0.2
3	36	19,737	1,071	91	0.1	0.7	0.3	0.0
4	2	696	48	2	0.0	0.0	0.0	0.0
5		23	1			0.0	0.0	
6		1				0.0		
	42,619	2,293,348	315,074	395,389				

Table 4. Ago2 Immunoprecipitation								
	Total Count				Percentage			
	siRNA I		siRNA II		siRNA I		siRNA II	
Number of U addition	AS	S	AS	S	AS	S	AS	S
0	973,951	297,275	252,018	226,015	91.0	88.4	91.0	73.4
1	95,649	30,650	22,829	70,611	8.9	9.1	8.2	22.9
2	833	7,758	2,053	10,255	0.1	2.3	0.7	3.3
3	63	611	96	1,030	0.0	0.2	0.0	0.3
4		12	6	15		0.0	0.0	0.0
5		1	1			0.0	0.0	0.0
6				1				0.0
	1,070,496	336,307	277,003	307,927				

Table 5. Dicer Immunoprecipitation								
	Total Count				Percentage			
	siRNA I		siRNA II		siRNA I		siRNA II	
Number of U addition	AS	S	AS	S	AS	S	AS	S
0	47,100	90,516	30,483	11,506	64.4	13.9	20.8	60.4
1	14,724	115,219	12,677	4,728	20.1	17.7	8.7	24.8
2	1,215	15,843	1,475	285	1.7	2.4	1.0	1.5
3	77	2,148	1	4		0.3	0.0	0.0
4		36				0.0		
5						0.0		
6						0.0		
	63,116	223,762	44,636	16,523				

[0085] The selected strands of siRNAs were truncated to the last nucleotide of 21 from total RNA (which is transfected siRNAs, collected total RNA and then sequenced). However, it disappeared non-selected strand of siRNAs of SI_AS and SII_S from total RNA (Figure 11, left). Surprisingly, the mono-uridylation predominantly exhibit in the sequencing cohort of Dicer bound siRNAs (Figure 12). No significant increment of mono-

uridylated siRNAs in Ago2 bound siRNAs was found (Figure 11, right). The uridylation pattern, including sequencing data from the total lysate, DICER immunoprecipitate, and Ago2 immunoprecipitate, was further investigated. The five most representative sequence variants of uridylated siRNAs were selected (Figure 13).

Example 4: Mono-uridylated siRNA affected by Dicer not Ago2

[0086] The impact of Dicer knockdown on uridylated siRNAs affected the activity of small RNA, indicating that Dicer may have affected efficiency of mono-uridylated siRNAs. However, the change of activity in siRNA and mono-uridylated siRNA was insignificant in Ago2-deficient cells; knockdown of Ago2 did not influence upon the activity of siRNA. Thus, Dicer may contribute to activity of mono-uridylated siRNAs (Figure 14A). Other siRNAs were further examined to detect the effect of mono-uridylation. To see whether other mono-uridylated siRNAs are regulated by the same mechanism with Dicer, the effects of mono-uridylation by other siRNAs of siRNA H1 and H2 were examined (Figure 14B). The sense strand of these selected siRNAs showed more gene knockdown efficiency than antisense strand (Figure 14C). M mono-uridylation was added in 3' end of the sense strand and gene knockdown efficiency was compared between original siRNAs and U added siRNAs in Dicer or Ago2 deleted cells. Another mono-uridylated siRNAs gave a similar but more dramatic result (Figure 14D). It was reported that Dicer can interact with many classes of RNA without endonuclease activity (Rybak-Wolf et al., 2014). This example indicates that mono-uridylated siRNAs promote, modulate, and/or regulate the activity of small RNA gene knockdown efficiency by Dicer processing.

Example 5: Eri1 affected different functions in miRNAs and siRNAs

[0087] Uridylation of siRNA might degrade with 3' to 5' exonuclease of Eri1. Therefore, the effect of human Eri1 was tested, using Eri-1 knockdown HCT116. Next, the miRNA abundance and/or distribution and uridylation bound with Dicer was tested. 3' uridylation of mature miRNA was reported to modulate the stability of small RNAs (Gutierrez-Vazquez et al., 2017). The normalized counts were significantly reduced in Dicer bound small RNAs. A global downregulation of miRNA was detected between negative control and Dicer bound miRNA when miRNA count distributions were analyzed with Kolmogorov Smirnoff test (Figures 15A and 15B). Uridylated miRNAs with Dicer were further evaluated (Figure 15C). The mean of the percentage of U addition in global

mature miRNA reduced in Dicer bound RNA (Figure 15D). On the other hand, the portion of uridylated siRNAs increased in Dicer immunoprecipitates (Figure 10). These results indicate that Dicer plays an integral role in the uridylation of miRNAs and siRNAs.

[0088] To determine the 3' exonuclease involved in miRNAs and siRNA biogenesis, Eri1 knockdown was generated by transfecting siRNA mixtures into HCT116 (Figure 16B) and deep sequencing was performed as Eri1 was reported to be a regulator of many types of RNA species and conduct microRNA homeostasis (Thomas et al., 2012) and histone mRNA. Besides, Eri1 forms a complex with Dicer that generates endo-siRNAs in Caenorhabditis elegans (Duchaine et al., 2006; Lee et al., 2006). The overall distribution of miRNAs upon global examination of the data decreased in siEri1 treated cells (Figure 17A). Surprisingly, a significant reduction of siRNA counts, both SI and SII, was observed in Eri1 defected cells (Figures 17B and 17C). The change was quantified by dividing the normalized read number of miRNAs with siEri1 by that of total miRNAs. The change was 100-fold in siRNA I (Figure 17C). Notably, the impact of Eri1 knockdown on the stability of small RNAs significantly differed between miRNAs and siRNAs, which indicated that Eri1 may have different roles in miRNAs and siRNAs.

[0089] Next, whether Eri1 had any effect on the uridylation of miRNAs and siRNA was determined. A global up-regulation of uridylated miRNA was detected in Eri1 mutants (Figure 17D). Interestingly, the abundance of uridylated siRNAs dramatically reduced in Eri1 deficient condition (Figure 17E). These data suggest that Eri1 can affect different pathway of uridylation for miRNAs and siRNAs.

Example 6: Potential mechanism of Eri1-mediated effects on smRNA stability

[0090] To understand the function of uridylation underlying Eri1 and Dicer in miRNAs, the relative miRNA expression levels were characterized by dividing the read number of miRNA with siEri1 by that of total miRNA(-control) [Log2(normalized counts of miRNAs in absent of Eri1/normalized counts of miRNA in total RNA fraction)] of an individual examination of each miRNA (Figure 18A). Five hundred forty-five miRNAs increased in Eri1 deficient cells.

[0091] In contrast, the total normalized counts of miRNAs in the siEri1 treated cell seemed to be decreased miRNAs when analyzed globally (Figure 17A). However, it was not shown the individual miRNA (Figure 18A). This inconsistency was because a global

analysis does not reflect the general behavior of each miRNA that is better that is characterized in the individual study.

[0092] The stability of many miRNAs (545 miRNAs) was increased in Eri1 defected cells (Figure 18A). But the normalized counts of siRNA were decreased in siEri1 (Figures 17B and 17C). Thus, Eri1 may have different impact on the stability of miRNAs and siRNAs.

[0093] A significant increment of miRNA uridylation was observed when treated with siEri1. The percentage of U addition of global miRNA was analyzed to investigate the change of miRNA upon knockdown of Eri1 (Figure 18B). The percentage of U addition in miRNAs increased after depletion of Eri1. The analysis of uridylation of individual miRNA revealed that many miRNAs (170 out of 253 uridylated miRNAs) uridylated in Eri1 depleted condition. These results strongly suggested that Eri1 inhibits most, if not all, miRNA uridylation.

[0094] If Eri1 exonuclease acts in separate pathways, the uridylated miRNAs should differ in up-regulated and down-regulated miRNAs by Eri1. The uridylation fraction of individual miRNA to grouped up-regulation (>1: more than 2-fold) or down-regulation (<-1: less than 2-fold) in Eri1-deleted cells depend on Figure 18A (Figure 18D). The group of up-regulated or down-regulated miRNA to collect the dots (each miRNA) in the right side include in the red bar increased the abundance in Eri1 knockdown cells (Figure 18E), which indicated that the grouped miRNA controlled the degradation by 3' to 5' exonuclease of Eri1. Down-regulated miRNAs may have coordinated the activation by Eri1. Previous studies showed that Eri1 was associated with both stabilization and degradation in histone mRNA biogenesis by controlling uridylation of mRNA (Scheer et al., 2016; Zuber et al., 2016). The percentage of uridylation was increased in the group of up-regulated miRNAs (Figure 18D). These results demonstrate that Eri1 was required for uridylation to control miRNA biogenesis.

[0095] To understand the mechanism uridylated miRNA biogenesis with Dicer, the uridylated miRNAs in Eri1 knockdown cells were reclassified into Dicer favor and disfavor. The expression level of miRNA bound to Dicer was determined by Dicer immunoprecipitation (Figure 15A). Dicer+ (Dicer favor) means that the expression of miRNA increased among Dicer bound small RNA (Figure 18F) and the cohort of Dicer

favor or disfavor miRNAs from each group of up or down regulated miRNAs by Eri1 was determined (Figure 18G). The grouping analysis revealed that the major of miRNAs (345 out of 545, 63%) preferred bind with Dicer in up-regulated miRNAs with siEri1, while down-regulated miRNAs (102 out of 147, 70%) in Eri1 knockout cells increased the population in the group of Dicer disfavor (Figure 18E). Thus, Dicer was necessary to maintain the stability of miRNAs depending on Eri1. To investigate the regulation of the uridylated miRNA depend on Dicer, the slope from comparable expression levels of each miRNA between HCT116 and Eri-depleted cells was carried out (Figures 18D and 18F). To correlate the uridylation with Dicer favored miRNAs and Dicer disfavored miRNAs, it was found that uridylation in siEri1 treated cells was highly positively correlated in Dicer positive group (Figures 18D and 18E). Also, a correlation between Dicer and Eri1 in group (cohort) of down-regulated miRNA was observed (Figure 18D). These results suggest that Eri1 and Dicer are related in uridylation mechanism.

Example 7: Cytidylated smRNAs stability

[0096] This example demonstrates that cytidylated smRNAs such as siRNAs have improved stability and can be used as therapeutic agents.

[0097] Similar to Example 3, the number of cytidine in the 3' end of siRNAs was analyzed and summarized in Table 6 below.

Table 6. C addition in the 3' end of siRNAs

	Total Count				Percentage			
Number of C addition	DsiRNA I	DsiRNA II	siRNA I	siRNA II	DsiRNA I	DsiRNA II	siRNA I	siRNA II
0	37,694	203,158	90,516	30,483	19.193	17.232	13.893	20.839
1	58,590	55,206	129,647	7,732	29.833	4.683	19.900	5.286
2	24,528	751,934	260,320	85,310	12.489	63.779	39.957	58.319
3	983	551	1,736	43	0.501	0.047	0.266	0.029
4	4	4	12		0.002	0.000	0.002	
5			5				0.001	
6			1				0.000	

Example 8: Effects of siRNAs on tumor model

[0098] Xenograft colon cancer model in balb-c mice was generated and treated with siRNA, CC-added siRNA and U added siRNA. The siRNAs were injected on Day 2.

Figure 50 and Table 7 show that the tumor volume decreased in subcutaneous injected U-added siRNA. However, C-added siRNA did not show too much effect, probably due to the spill of liquid after injection.

Table 7. Tumor reduction by siRNAs

		siRNA	Day 1	Day 2	Tumor volume Day 4	
1	Male_No_P	siRNA_U [142 ng/g]	0.0539	0.625	0.6*0.6	0.108
2	Female_1P_L	siRNA	0.216	0.216	1.2*1.5	1.08
3	Female_1P_R	siRNA_CC	0.294	1.008	1.5*2	2.25
4	Female_2P_L	siRNA_U	0.294	0.567	1.5*0.8	0.48
5	Female_2P_R	saline	0.368	0.907	2*1.3	1.69

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CLAIMS

1. A method of improving stability of a small RNA molecule comprising adding one or more non-templated nucleotides to the 3' end of the small RNA molecule.

2. The method of claim 1, comprising contacting the small RNA molecule with a 3' to 5' exonuclease to remove 3' overhang before adding the one or more non-templated nucleotides.

3. The method of claim 1 or claim 2, wherein one, two, three, four, or five non-templated nucleotides are added to the 3' end of the small RNA molecule.

4. The method of any one of claims 1-3, wherein the non-templated nucleotide is cytidine.

5. The method of any one of claims 1-3, wherein the non-templated nucleotide is uridine.

6. The method of any one of claims 1-5, wherein the small RNA molecule is an siRNA, an shRNA or a miRNA.

7. The method of any one of claims 1-6, wherein the small RNA molecule is derived from Dicer.

8. The method of any one of claims 1-7, wherein the small RNA molecule has a size of less than 50 bps, less than 45 bps, less than 40 bps, less than 35 bps, less than 30 bps, less than 35 bps, less than 30 bps, less than 25 bps, less than 20 bps, or less than 15 bps.

9. The method of any one of claims 1-8, wherein the small RNA molecule has a size of about 15 bps, about 16 bps, about 17 bps, about 18 bps, about 19 bps, about 20 bps, about 21 bps, about 22 bps, about 23 bps, about 24 bps, about 25 bps, about 26 bps, about 27 bps, about 28 bps, about 29 bps, or about 30 bps.

10. A modified small RNA molecule comprising the nucleotide sequence of an

unmodified small RNA molecule, and one or more non-templated nucleotides at the 3' end of the unmodified small RNA molecule, wherein the modified small RNA molecule has an improved *in vivo* stability relative to the unmodified small RNA molecule.

11. The modified small RNA molecule of claim 10, comprising one, two, three, four, or five non-templated nucleotides at the 3' end of the unmodified small RNA molecule.

12. The modified small RNA molecule of claim 10 or claim 11, wherein the non-templated nucleotide is cytidine.

13. The modified small RNA molecule of claim 10 or claim 11, wherein the non-templated nucleotide is uridine.

14. The modified small RNA molecule of any one of claims 10-13, wherein the small RNA molecule is an siRNA, an shRNA or a miRNA.

15. The modified small RNA molecule of any one of claims 10-14, wherein the small RNA molecule is derived from Dicer.

16. The modified small RNA molecule of any one of claims 10-15, wherein the small RNA molecule has a size of less than 50 bps, less than 45 bps, less than 40 bps, less than 35 bps, less than 30 bps, less than 35 bps, less than 30 bps, less than 25 bps, less than 20 bps, or less than 15 bps.

17. The modified small RNA molecule of any one of claims 10-16, wherein the small RNA molecule has a size of about 15 bps, about 16 bps, about 17 bps, about 18 bps, about 19 bps, about 20 bps, about 21 bps, about 22 bps, about 23 bps, about 24 bps, about 25 bps, about 26 bps, about 27 bps, about 28 bps, about 29 bps, or about 30 bps.

18. A pharmaceutical composition comprising the modified small RNA molecule of any one of claims 10-17.

19. A method of treating a disease or condition selected from cancer, a

physiological and metabolic disorder, and a viral infection comprising administering to a subject suffering such a disease or condition a therapeutically effective amount of the modified small RNA molecule of any one of claims 10-17 or the pharmaceutical composition of claim 18.

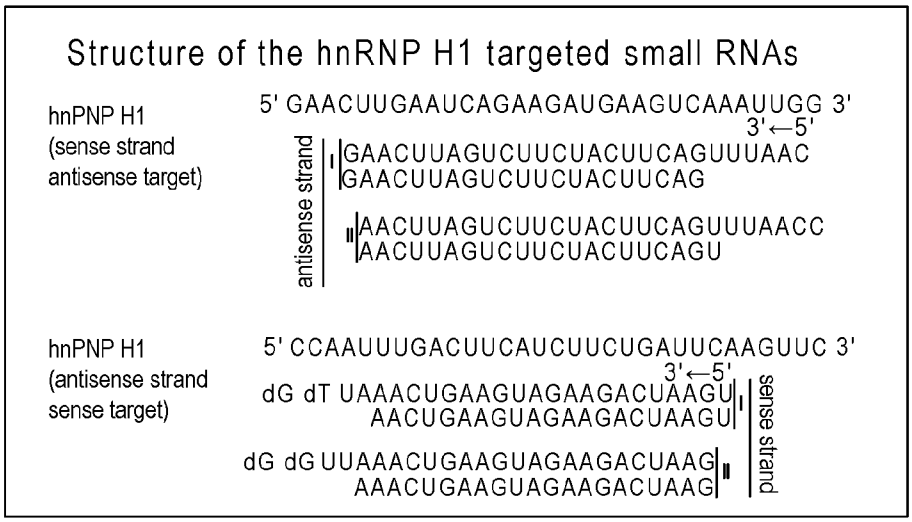

Structure of the hnRNP H1 targeted small RNAs
hnPNP H1
(sense strand
antisense target)
5' GAACUUGAAUCAGAAGAUGAAGUCAAAUUGG 3'
3'←5'
antisense strand
GAACUUAGUCUUCUACUUCAGUUUAAC
GAACUUAGUCUUCUACUUCAG
AACUUAGUCUUCUACUUCAGUUUAACC
AACUUAGUCUUCUACUUCAGU
hnPNP H1
(antisense strand
sense target)
5' CCAAUUUGACUUCAUCUUCUGAUUCAAGUUC 3'
3'←5'
dG dT UAAACUGAAGUAGAAGACUAAGU
AACUGAAGUAGAAGACUAAGU
dG dG UUAAACUGAAGUAGAAGACUAAG
AAACUGAAGUAGAAGACUAAG
sense strand

FIG. 1

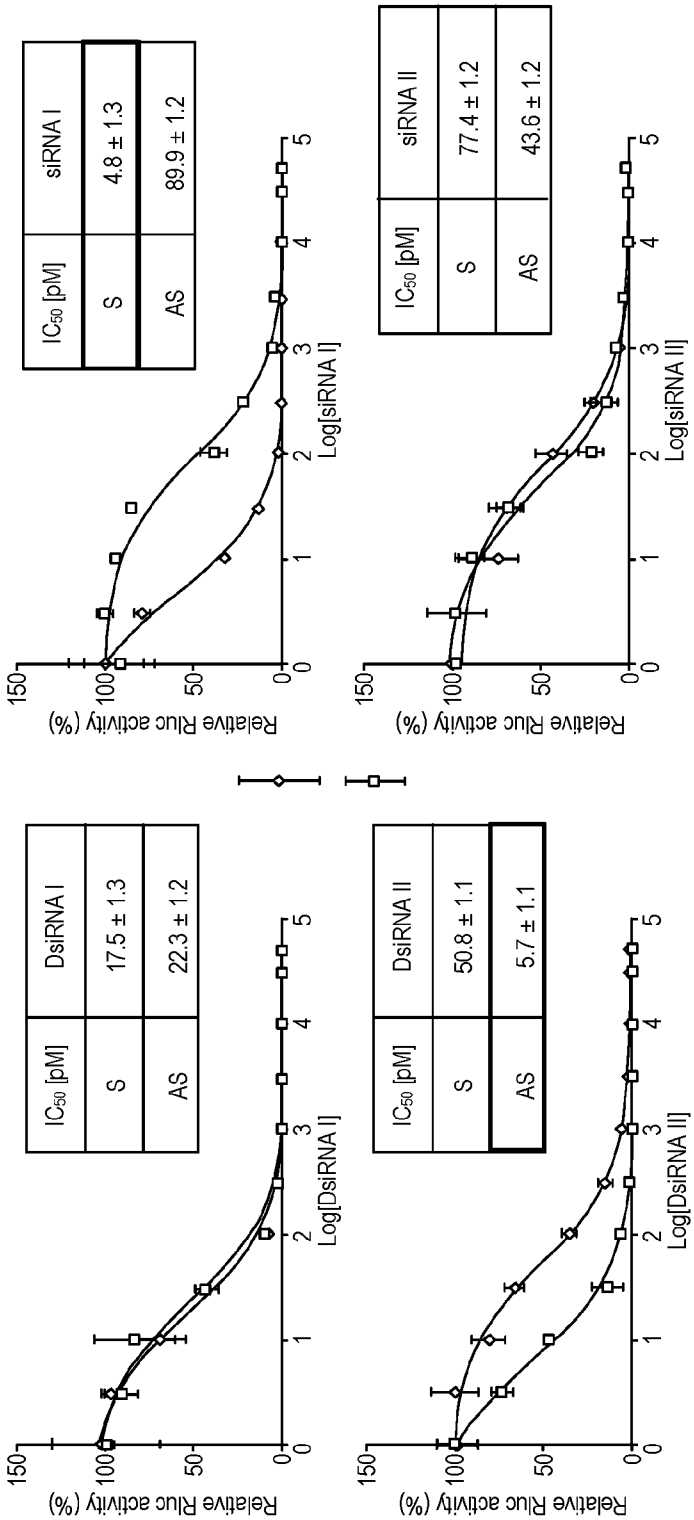

IC50 [pM] | siRNA I
S | 4.8 ± 1.3
AS | 89.9 ± 1.2
IC50 [pM] | siRNA II
S | 77.4 ± 1.2
AS | 43.6 ± 1.2
IC50 [pM] | DsiRNA I
S | 17.5 ± 1.3
AS | 22.3 ± 1.2
IC50 [pM] | DsiRNA II
S | 50.8 ± 1.1
AS | 5.7 ± 1.1
Relative Rluc activity (%)
Log[siRNA I]
Log[siRNA II]
Log[DsiRNA I]
Log[DsiRNA II]

FIG. 2

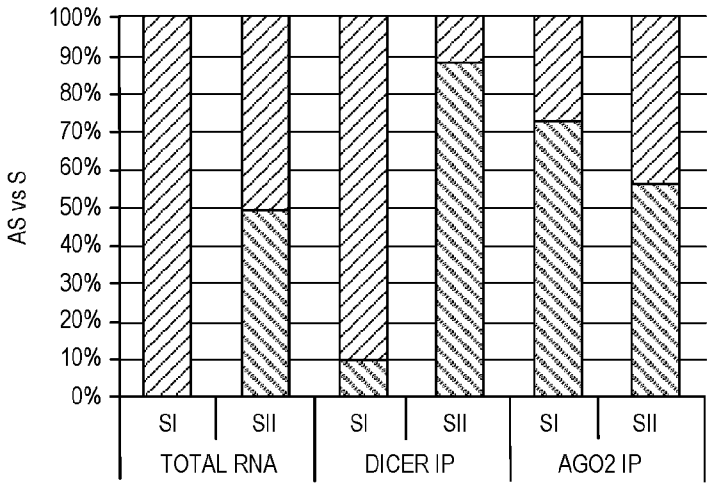

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
AS vs S
SI
SII
SI
SII
SI
SII
TOTAL RNA
DICER IP
AGO2 IP

FIG. 3

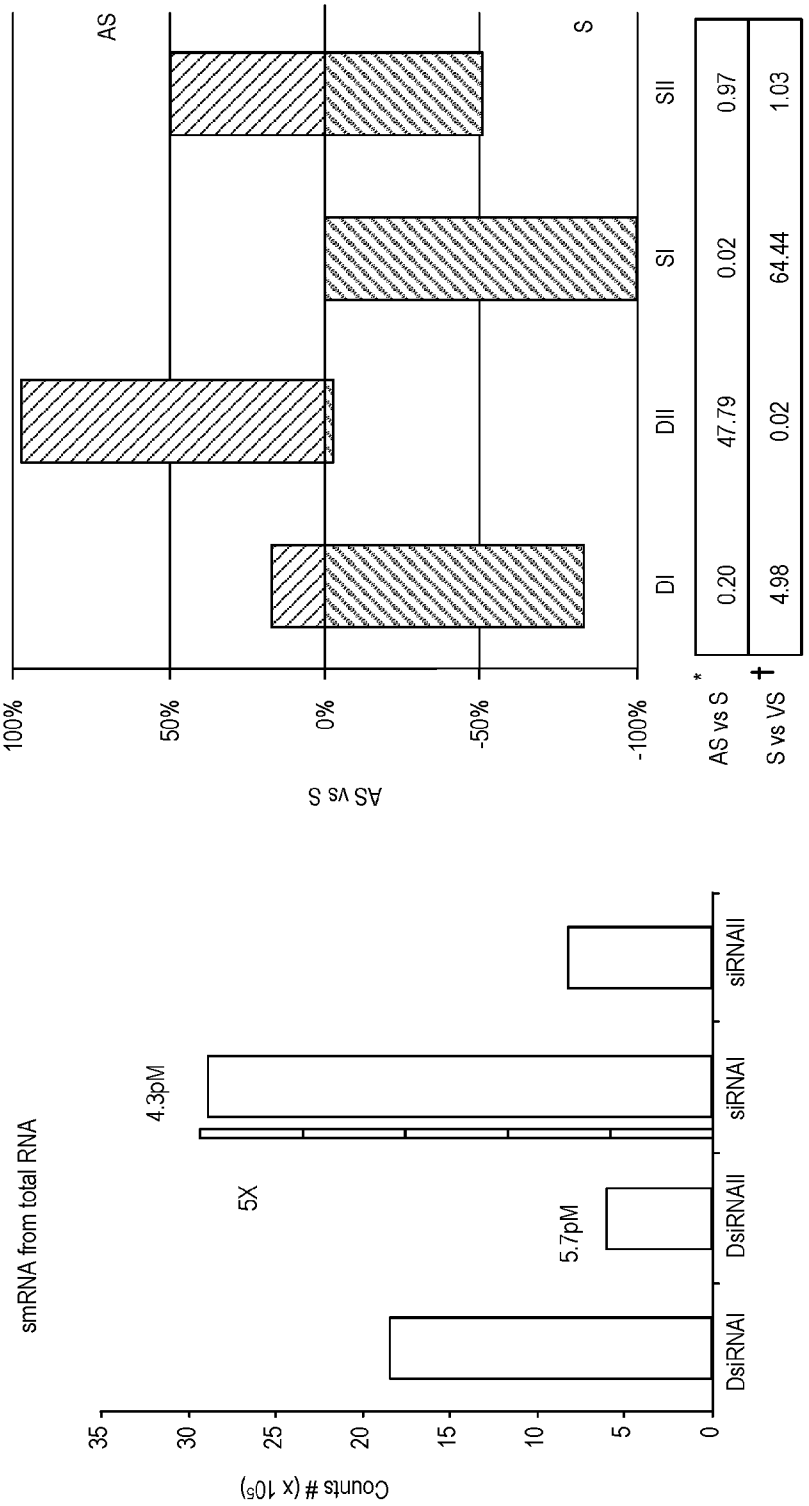

smRNA from total RNA
Counts # (x 10⁵)
35
30
25
20
15
10
5
0
DsiRNAI
DsiRNAII
siRNAI
siRNAII
4.3pM
5X
5.7pM
AS vs S
100%
50%
0%
-50%
-100%
AS
S
DI
DII
SI
SII
AS vs S
S vs VS
0.20
47.79
0.02
0.97
4.98
0.02
64.44
1.03

FIG. 4

		DsiRNAI	DsiRNA II	siRNA I	siRNA II
EFFICIENCY	S	17.5 pM	50.8 pM	4.3 pM	77.4 pM
	AS	22.3 pM	5.7 pM	89.9 pM	44.6 pM
STABILITY	S	153,127	127,114	2,853,298	419,135
	AS	309,404	591,709	45,542	405,642

0.6pM

5X

6X

FIG. 5

		DsiRNA II		siRNA II	
EFFICIENCY	AS	5.7 pM	>	44.6 pM	⇨ Selected strand
STABILITY	AS	591,709		405,642	

		DsiRNA I		siRNA I	
EFFICIENCY	AS	22.3 pM	>	89.9 pM	⇨ Non-selected strand
STABILITY	AS	309,404		45,542	

FIG. 6

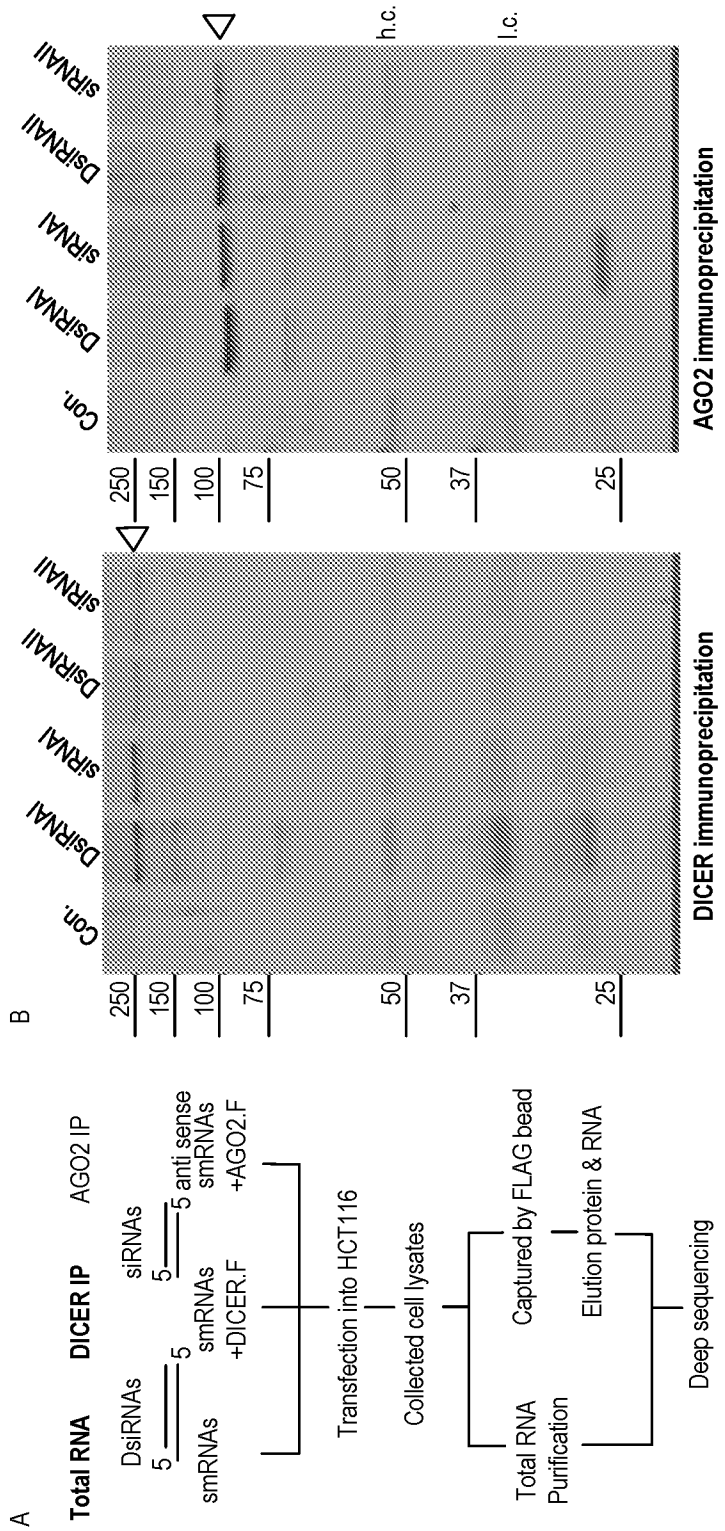

A
Total RNA
DICER IP
AGO2 IP
DsiRNAs
siRNAs
5
5
5
5
anti sense
smRNAs
smRNAs
smRNAs
+DICER.F
+AGO2.F
Transfection into HCT116
Collected cell lysates
Total RNA
Purification
Captured by FLAG bead
Elution protein & RNA
Deep sequencing
B
Con.
DsiRNAi
siRNAi
DsiRNAii
siRNAii
250
150
100
75
50
37
25
h.c.
l.c.
DICER immunoprecipitation
AGO2 immunoprecipitation

FIG. 7

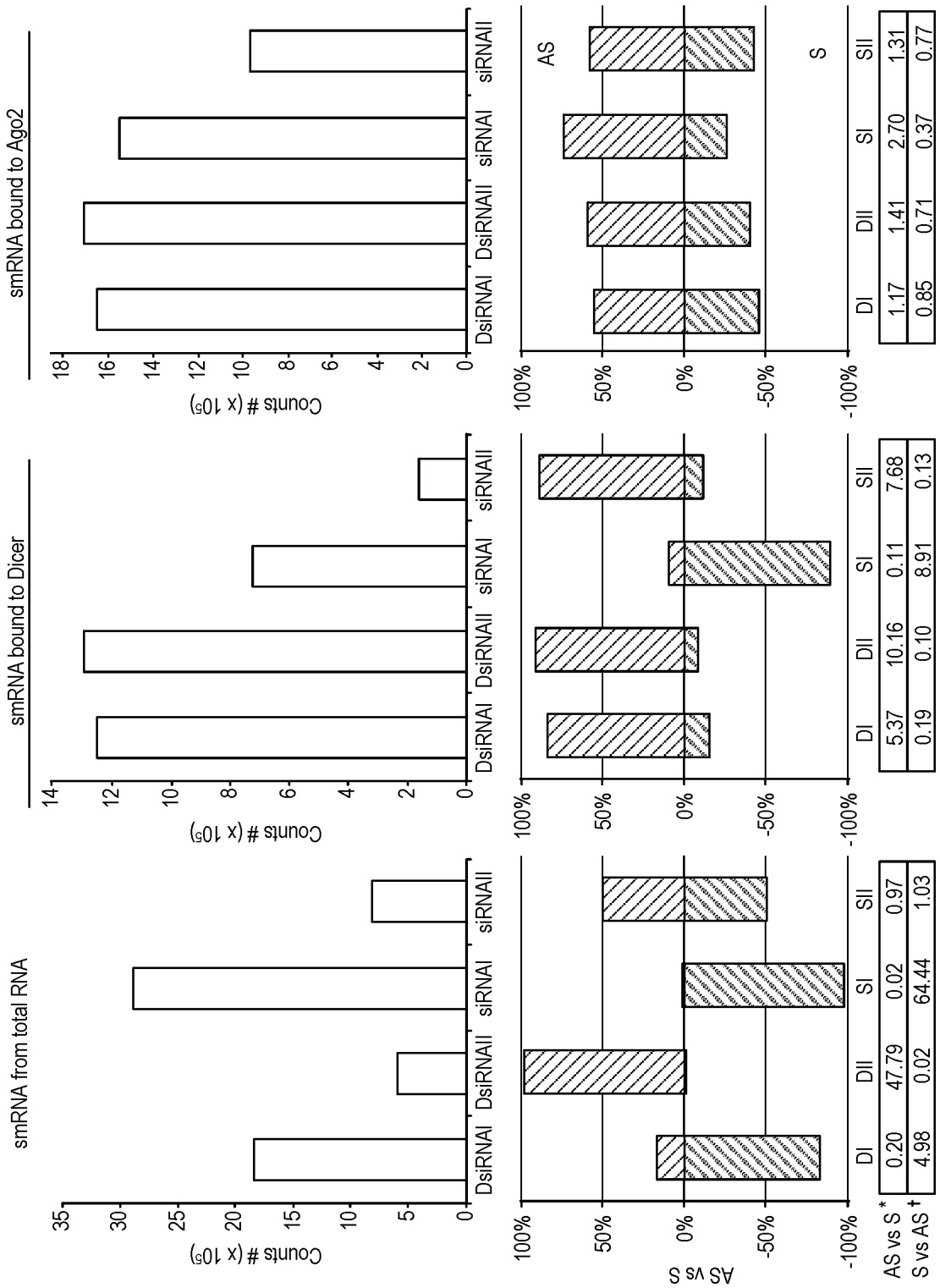

smRNA from total RNA
smRNA bound to Dicer
smRNA bound to Ago2
Counts # (x 10⁵)
DsiRNAI
DsiRNAII
siRNAI
siRNAII
AS
S
AS vs S
DI
DII
SI
SII
AS vs S *
S vs AS †
0.20 47.79 0.02 0.97
4.98 0.02 64.44 1.03
5.37 10.16 0.11 7.68
0.19 0.10 8.91 0.13
1.17 1.41 2.70 1.31
0.85 0.71 0.37 0.77

FIG. 8

DsiRNA II_antisense

Sequence CCAATTTGACTTGATCTTCTGATTCAA		Total RNA Count	%		Ago2 IP Count	%		DICER IP Count	%
TTTGACTTCATCTTCTGATTCA	1st	110,274	19	1st	415,702	41			
TTGACTTCATCTTCTGATTCA	2nd	106,171	18	3rd	67,380	7			
TGACTTCATCTTCTGATTCA	3rd	95,791	16	2nd	75,155	7			
TGACTTCATCTTCTGATTCAA	4th	46,645	8						
TTGACTTCATCTTCTGATTCAA	5th	32,259	5						
TTTGACTTCATCTTCTGATTC	6th	18,226	3						
TTGACTTCATCTTCTGATTC	7th	16,565	3						
TGACTTCATCTTCTGATTCACC	8th	12,484	2				1st	646,127	55
TGACTTCATCTTCTGATTCAAT	9th	10,398	2				2nd	91,563	8
TTTGACTTCATCTTCTGATTCAA	10th	9,910	2						

DsiRNA II_sense

Sequence GAATCAGAAGATGAAGTCAAATTdGdG		Total RNA Count	%		Ago2 IP Count	%		DICER IP Count	%
GAATCAGAAGATGAAGTCA	1st	1,576	16						
GAATCAGAAGATGAAGTCAA	2nd	1,203	12	3rd	77,915	15			
GAATCAGAAGATGAAGTCAAAT	3rd	1,125	11	2nd	153,713	29			
GAATCAGAAGATGAAGTCAAT	4th	1,037	10				1st	29,332	25
GAATCAGAAGATGAAGTCAAATTdGdG	5th	1,019	10						
GAATCAGAAGATGAAGTCAAA	6th	1,019	10						
AGAAGATGAAGTCAAA	7th	808	8						
GAATCAGAAGATGAAGTCAAATT	8th	470	5	1st	179,240	34			
AGAAGATGAAGTCAAATT	9th	356	4						
AGAAGATGAAGTCAA	10th	352	3						

FIG. 9A

siRNA I_antisense

Sequence GACTTCATCTTCTGATTCAAG		Total RNA Count	%		Ago2 IP Count	%		DICER IP Count	%
GACTTCATCTTCTGATTCAAG	1st	11,413	25	1st	847,541	73	1st	23,793	33
ACTTCATCTTCTGATTCAAG	2nd	7,354	16	2nd	78,062	7			
CTTCATCTTCTGATTCAAG	3rd	5,351	12						
CATCTTCTGATTCAAG	4th	3,334	7						
GACTTCATCTTCTGATTCA	5th	2,581	6						
GACTTCATCTTCTGATTCAAGT	6th	2,374	5	3rd	71,147	6	3rd	5,798	8
GACTTCATCTTCTGATTCAA	7th	1,611	4						
GACTTCATCTTCTGATTCAAT	8th	1,390	3				2nd	6,791	9
ATCTTCTGATTCAAG	9th	1,189	3						
TCATCTTCTGATTCAAG	10th	1,133	2						

siRNA I_sense

Sequence TGAATCAGAAGATGAAGTCAA		Total RNA Count	%		Ago2 IP Count	%		DICER IP Count	%
TGAATCAGAAGATGAAGTCA	1st	1,158,408	41	2nd	113,639	27			
TGAATCAGAAGATGAAGTCAA	2nd	433,899	15	1st	163,953	39			
TGAATCAGAAGATGAAGTCAAT	3rd	240,204	8				2nd	101,685	16
TGAATCAGAAGATGAAGTCAC	4th	236,612	8						
TGAATCAGAAGATGAAGTCACC	5th	222,876	8	3rd	29,375	7	1st	245,458	38
TGAATCAGAAGATGAAGTC	6th	113,402	4						
TGAATCAGAAGATGAAGTCAATT	7th	98,894	3						
TGAATCAGAAGATGAAGTCACT	8th	73,209	3						
TGAATCAGAAGATGAAGTCAT	9th	68,772	2						
TGAATCAGAAGATGAAGTCACCA	10th	49,440	2						

FIG. 9B

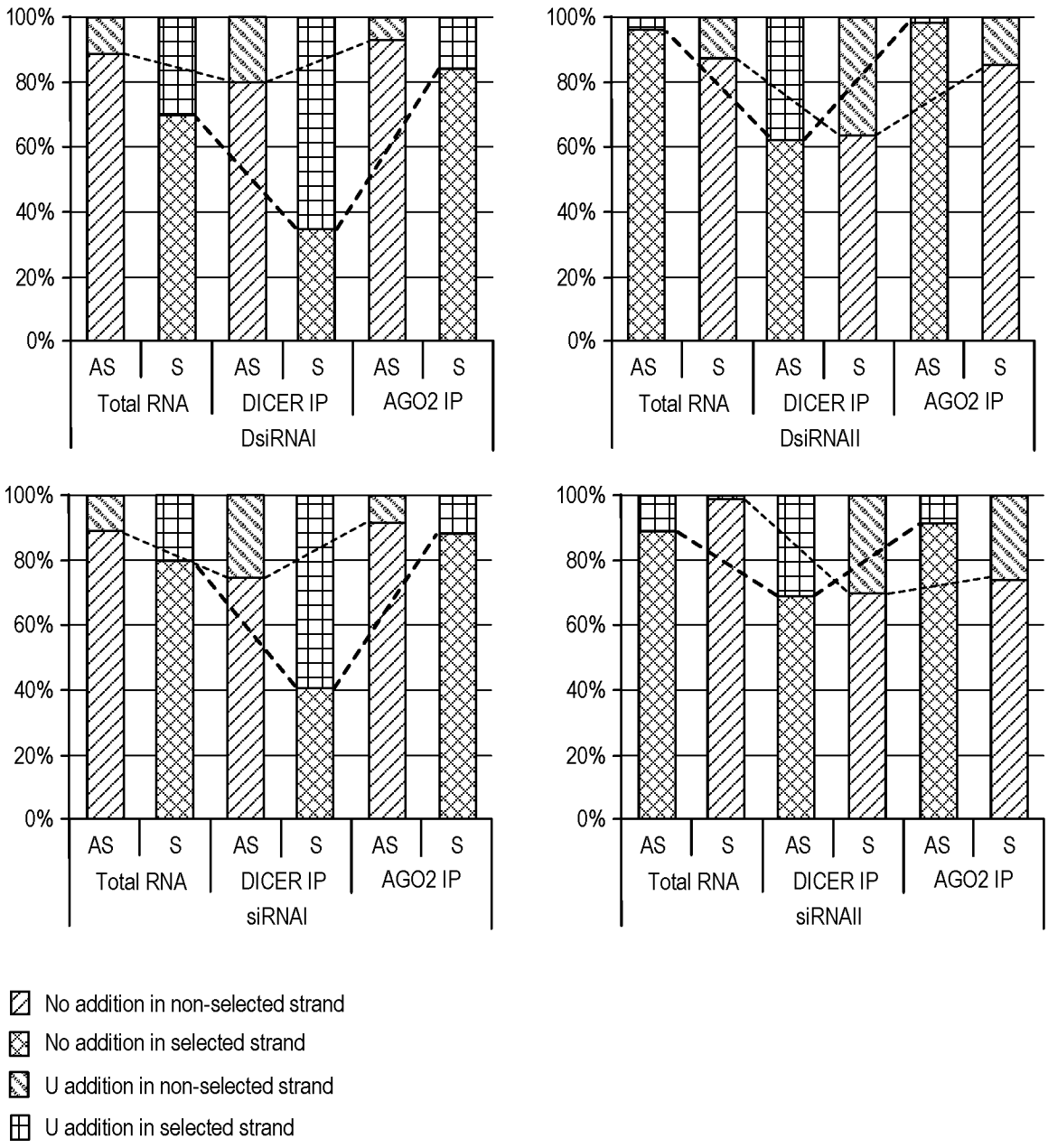

100%
80%
60%
40%
20%
0%
AS
S
Total RNA
DICER IP
AGO2 IP
DsiRNAI
DsiRNAII
siRNAI
siRNAII
No addition in non-selected strand
No addition in selected strand
U addition in non-selected strand
U addition in selected strand

FIG. 10

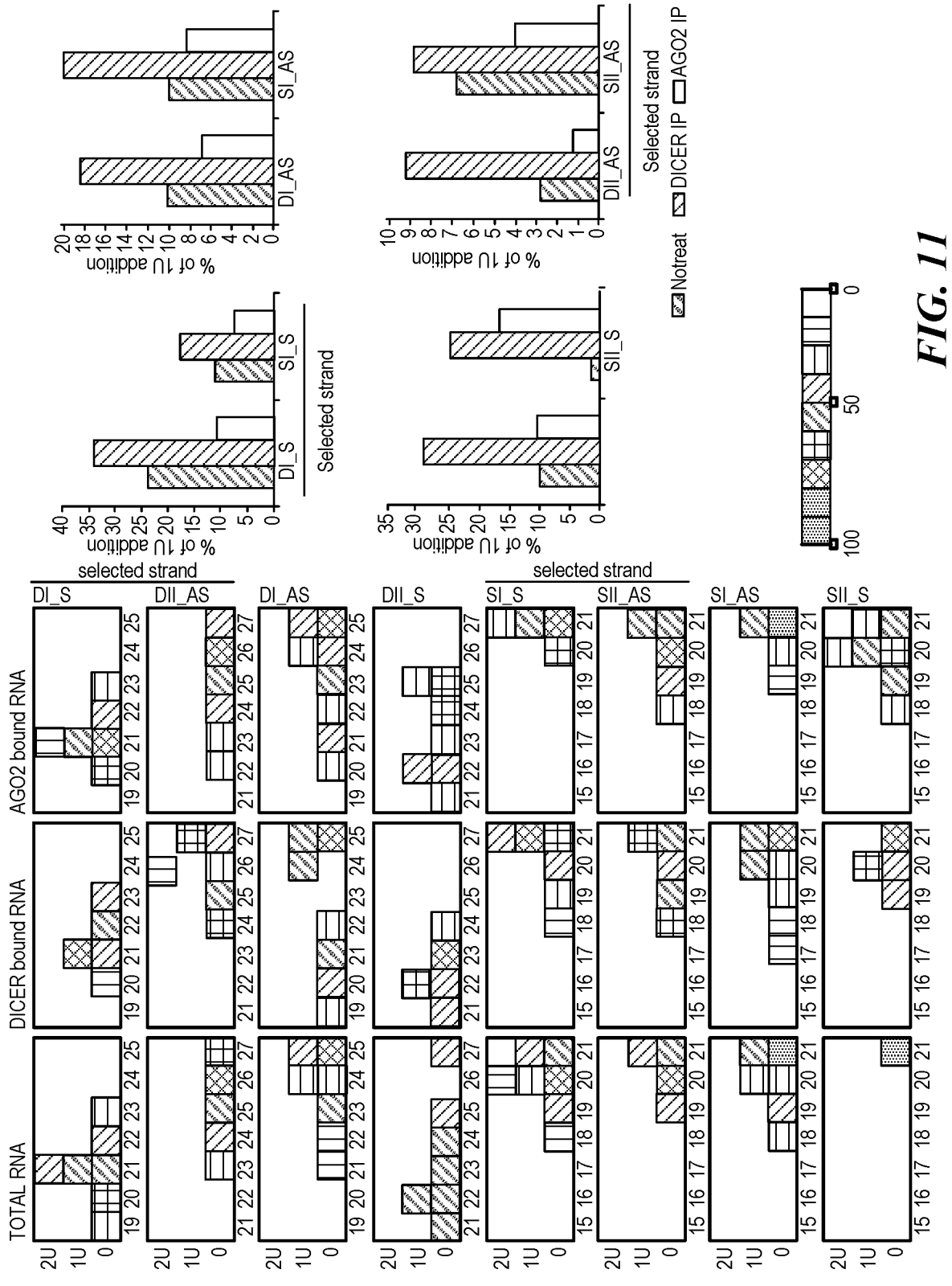

TOTAL RNA
DICER bound RNA
AGO2 bound RNA
selected strand
DI_S
DII_AS
DI_AS
DII_S
SI_S
SII_AS
SI_AS
SII_S
2U
1U
0
% of 1U addition
Selected strand
Notreat
DICER IP
AGO2 IP
100
50
0

FIG. 11

S: Sense strand
AS : Anti-sense strand
siRNA I
UGAAUCAGAAGAUGAAGUCAA
GACUUCAUCUUCUGAUUCAAG
346,383 / 471,936 (73%)
2,453 / 4,925 (50%)
siRNA II
GAAUCAGAAGAUGAAGUCAAA
UGACUUCAUCUUCUGAUUCAA
6,127 / 6,608 (93%)
26,384 / 35,843 (74%)

FIG. 12

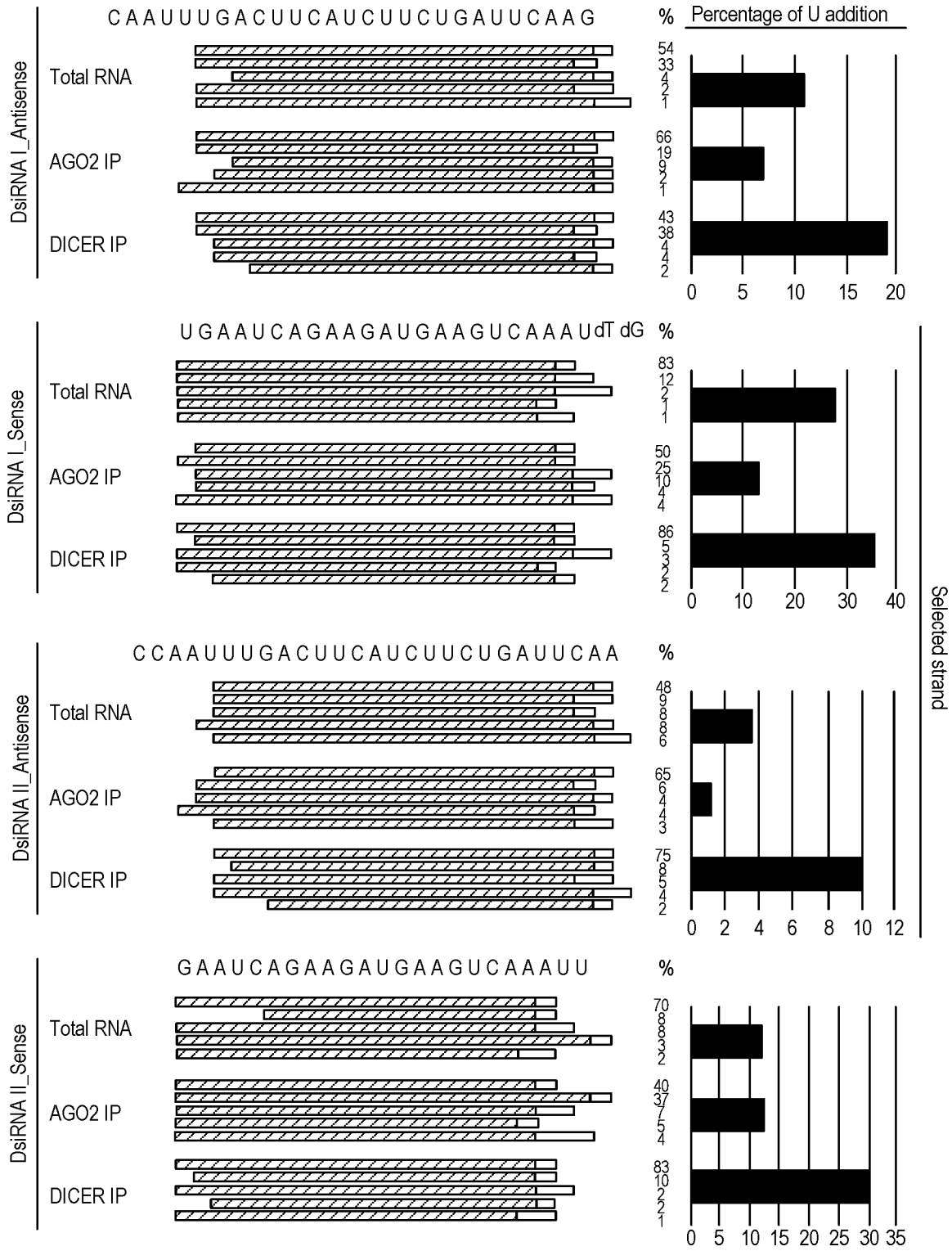

DsiRNA I_Antisense
C A A U U U G A C U U C A U C U U C U G A U U C A A G
Total RNA
AGO2 IP
DICER IP
%
Percentage of U addition
54 33 4 2 1
66 19 9 2 1
43 38 4 4 2
0 5 10 15 20
DsiRNA I_Sense
U G A A U C A G A A G A U G A A G U C A A A U dT dG
Total RNA
AGO2 IP
DICER IP
%
83 12 2 1 1
50 25 10 4 4
86 5 3 2 2
0 10 20 30 40
DsiRNA II_Antisense
C C A A U U U G A C U U C A U C U U C U G A U U C A A
Total RNA
AGO2 IP
DICER IP
%
48 9 8 8 6
65 6 4 4 3
75 8 5 4 2
0 2 4 6 8 10 12
DsiRNA II_Sense
G A A U C A G A A G A U G A A G U C A A A U U
Total RNA
AGO2 IP
DICER IP
%
70 8 8 3 2
40 37 7 5 4
83 10 2 2 1
0 5 10 15 20 25 30 35
Selected strand

FIG. 13

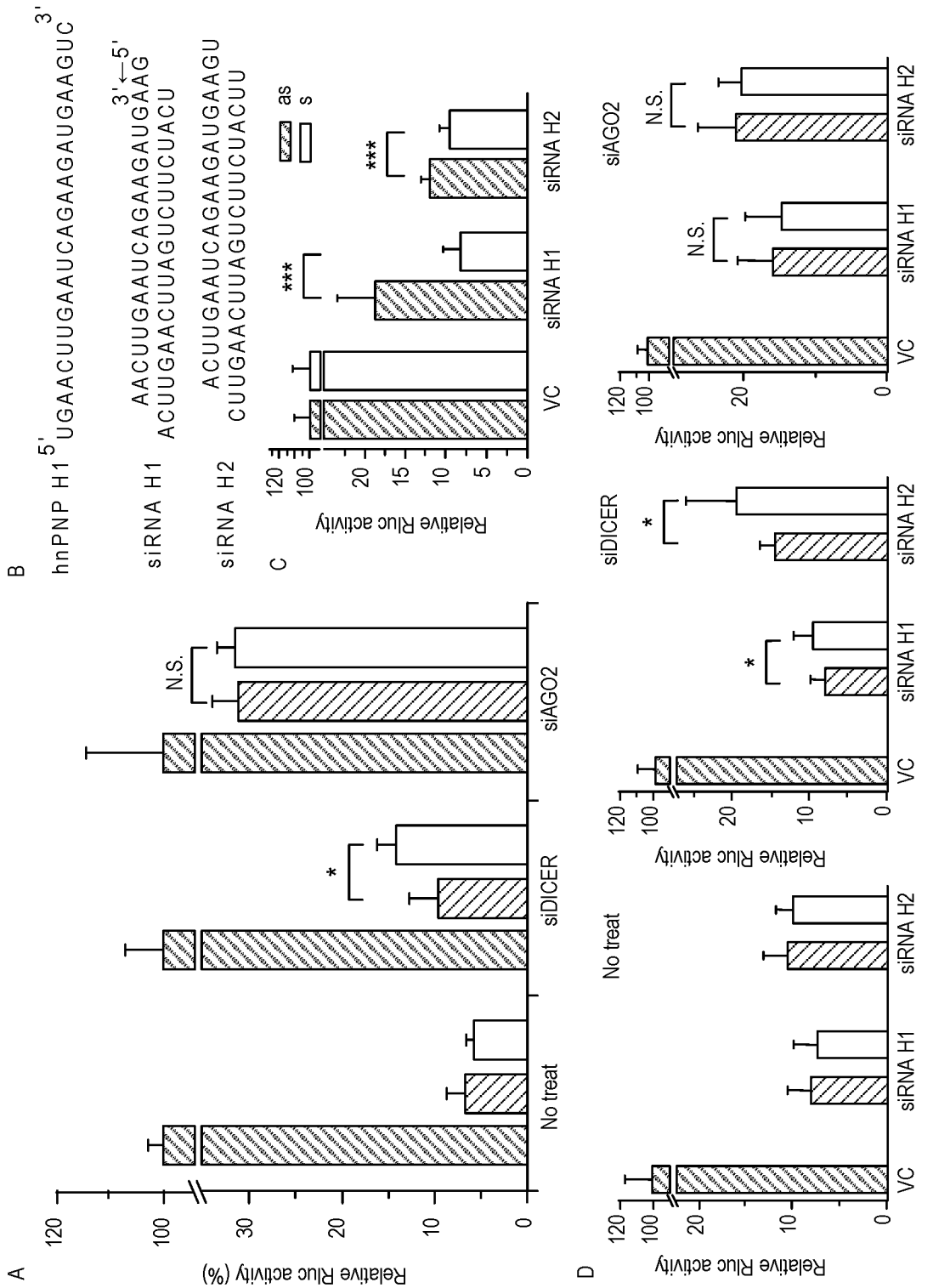

A
Relative Rluc activity (%)
120
100
30
20
10
0
No treat
siRNA H1
siRNA H2
siDICER
siAGO2
N.S.
*
B
hnPNP H1 5' UGAACUUGAAUCAGAAGAUGAAGUC 3'
siRNA H1 AACUUGAAUCAGAAGAUGAAG 3'←5'
ACUUGAACUUAGUCUUCUACU
siRNA H2 ACUUGAAUCAGAAGAUGAAGU
CUUGAACUUAGUCUUCUACUU
C
Relative Rluc activity
120
100
20
15
10
5
0
as
s
***
***
VC
siRNA H1
siRNA H2
D
Relative Rluc activity
120
100
20
10
0
VC
siRNA H1
siRNA H2
No treat
siDICER
siAGO2
*
*
N.S.
N.S.

FIG. 14

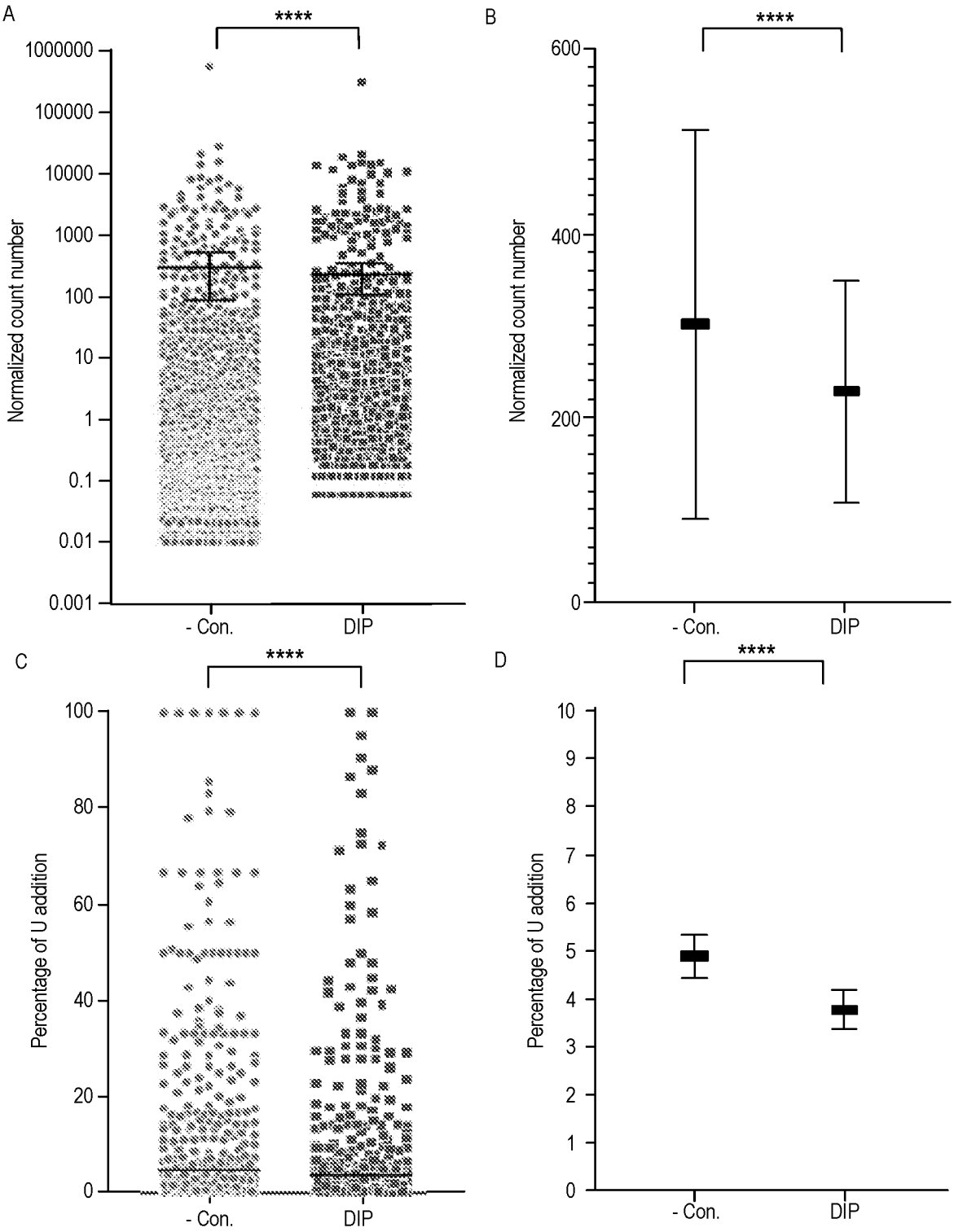

A
****
1000000
100000
10000
1000
100
10
1
0.1
0.01
0.001
Normalized count number
- Con.
DIP
B
****
600
400
200
0
Normalized count number
- Con.
DIP
C
****
100
80
60
40
20
0
Percentage of U addition
- Con.
DIP
D
****
10
9
8
7
6
5
4
3
2
1
0
Percentage of U addition
- Con.
DIP

FIG. 15

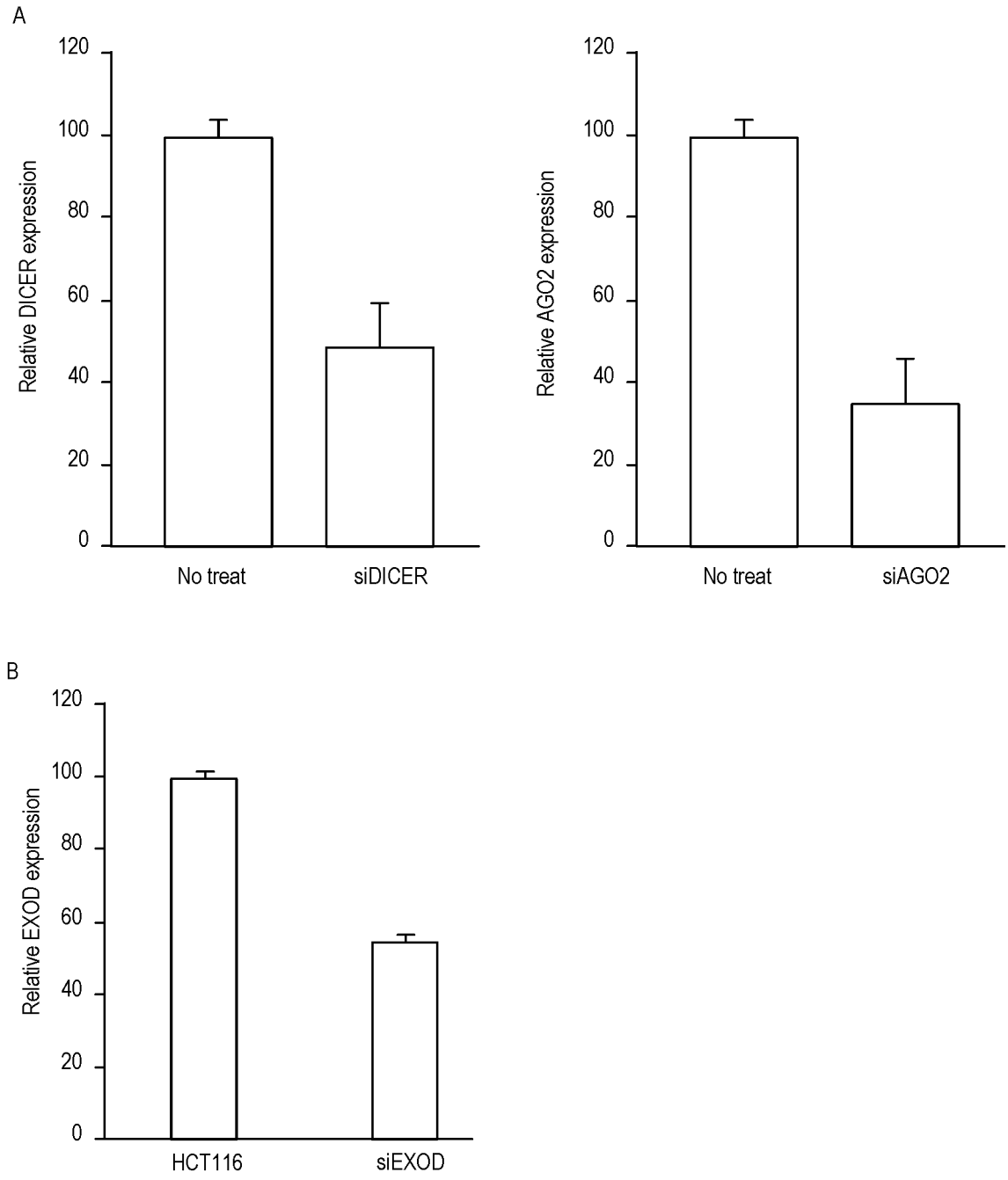

A
120
100
80
60
40
20
0
Relative DICER expression
No treat
siDICER
120
100
80
60
40
20
0
Relative AGO2 expression
No treat
siAGO2
B
120
100
80
60
40
20
0
Relative EXOD expression
HCT116
siEXOD

FIG. 16

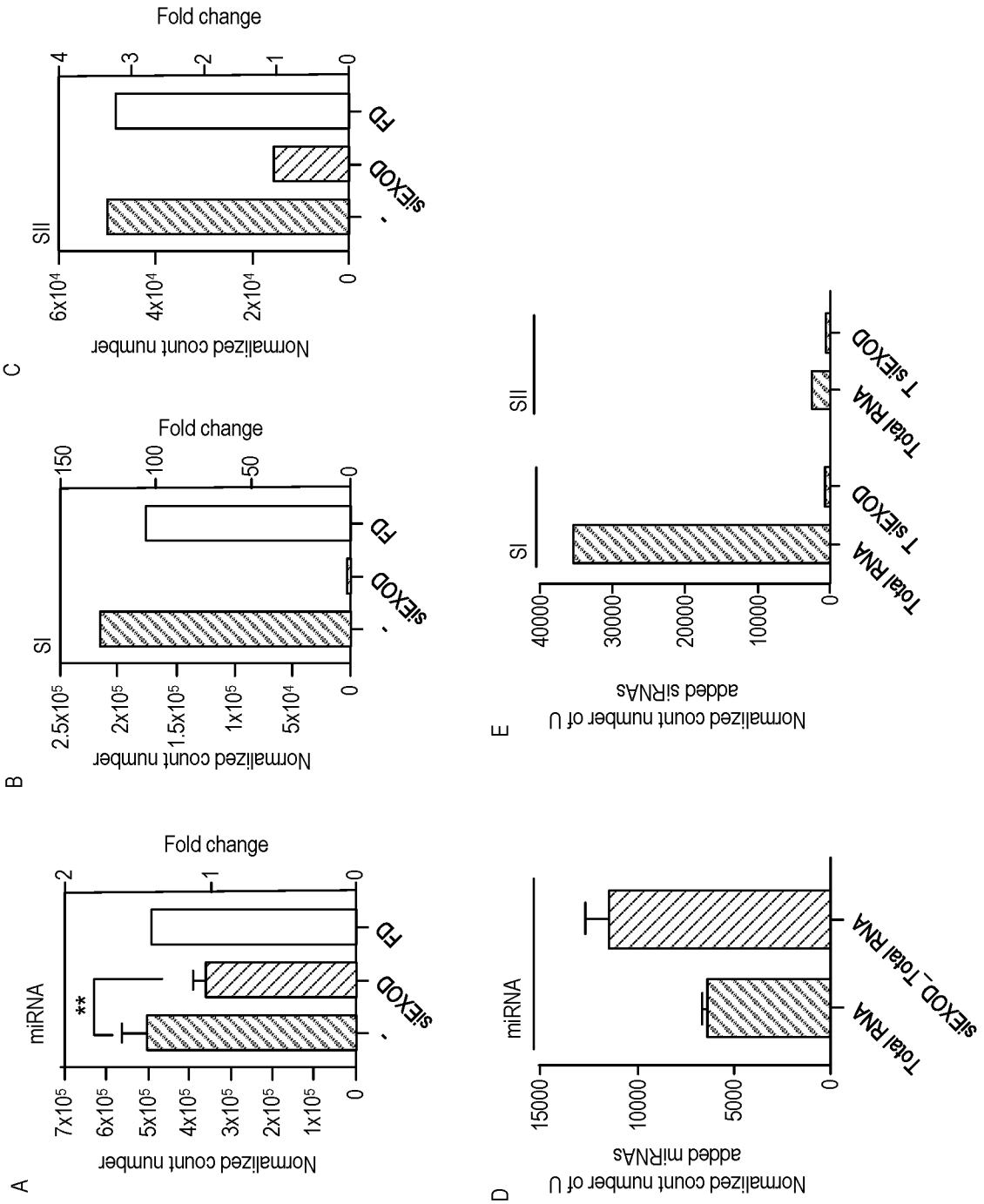

A
Normalized count number
miRNA
Fold change
**
-
siEXOD
FD
B
Normalized count number
SI
Fold change
-
siEXOD
FD
C
Normalized count number
SII
Fold change
-
siEXOD
FD
D
Normalized count number of U added miRNAs
miRNA
Total RNA
siEXOD_Total RNA
E
Normalized count number of U added siRNAs
SI
SII
Total RNA
T siEXOD
Total RNA
T siEXOD

FIG. 17

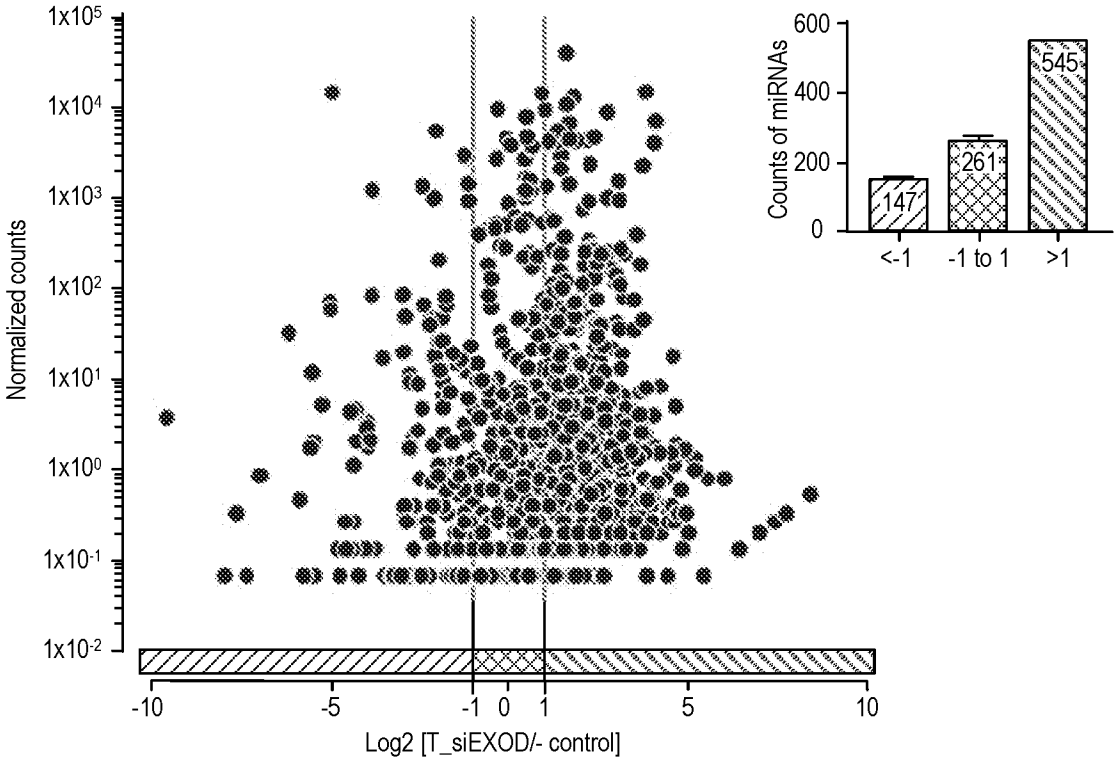


FIG. 18A

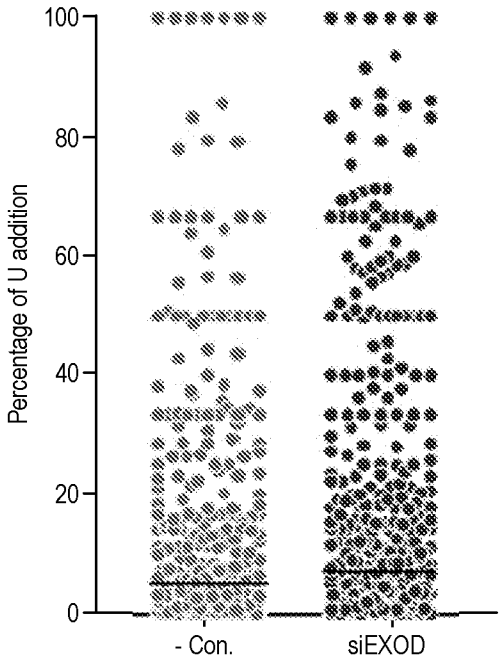


FIG. 18B

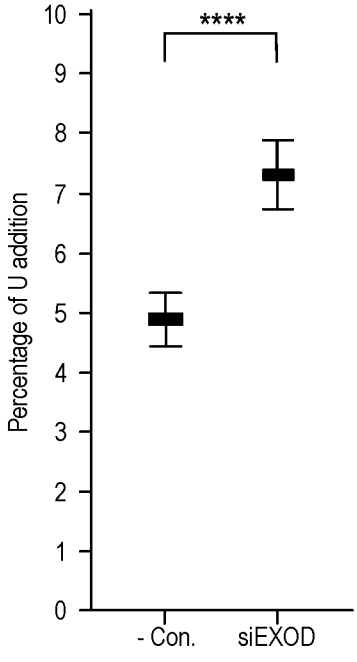


FIG. 18C

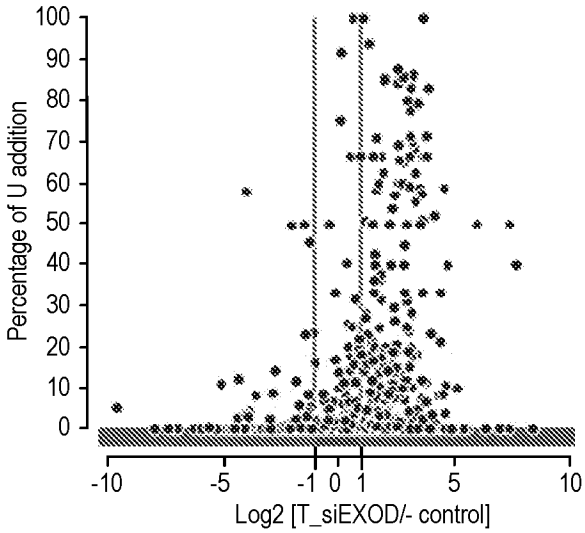


FIG. 18D

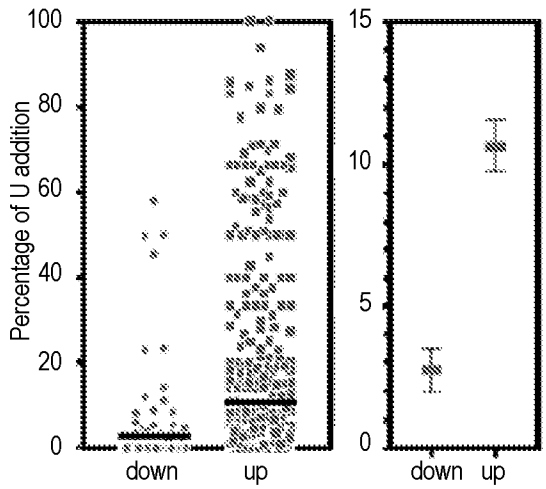


FIG. 18E

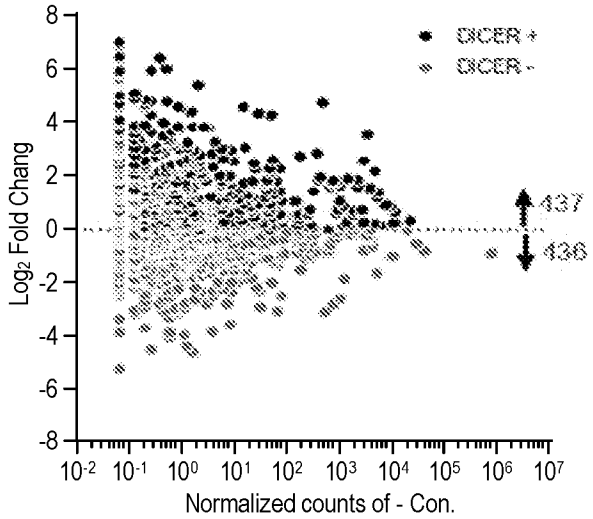


FIG. 18F

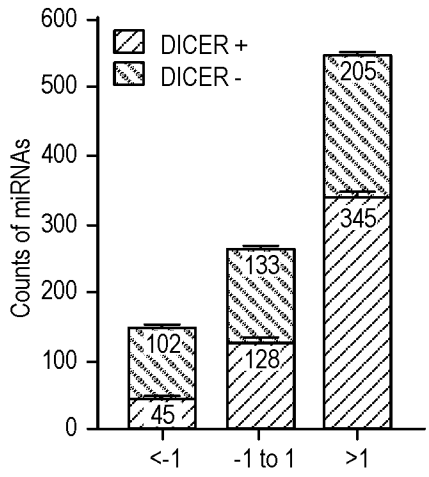


FIG. 18G

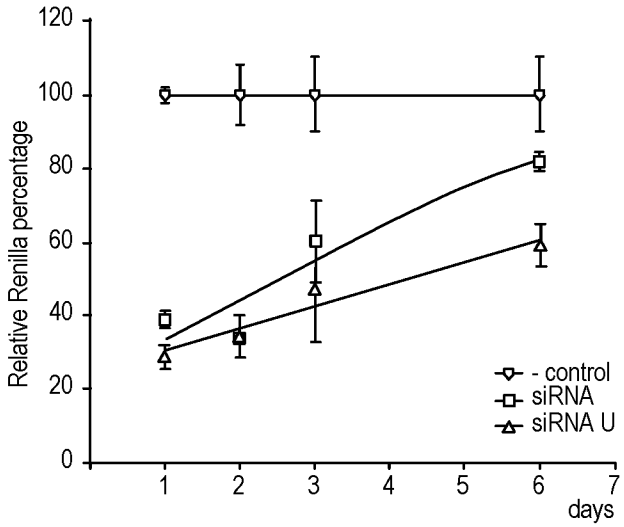

120
100
80
60
40
20
0
Relative Renilla percentage
1
2
3
4
5
6
7
days
- control
siRNA
siRNA U

FIG. 19

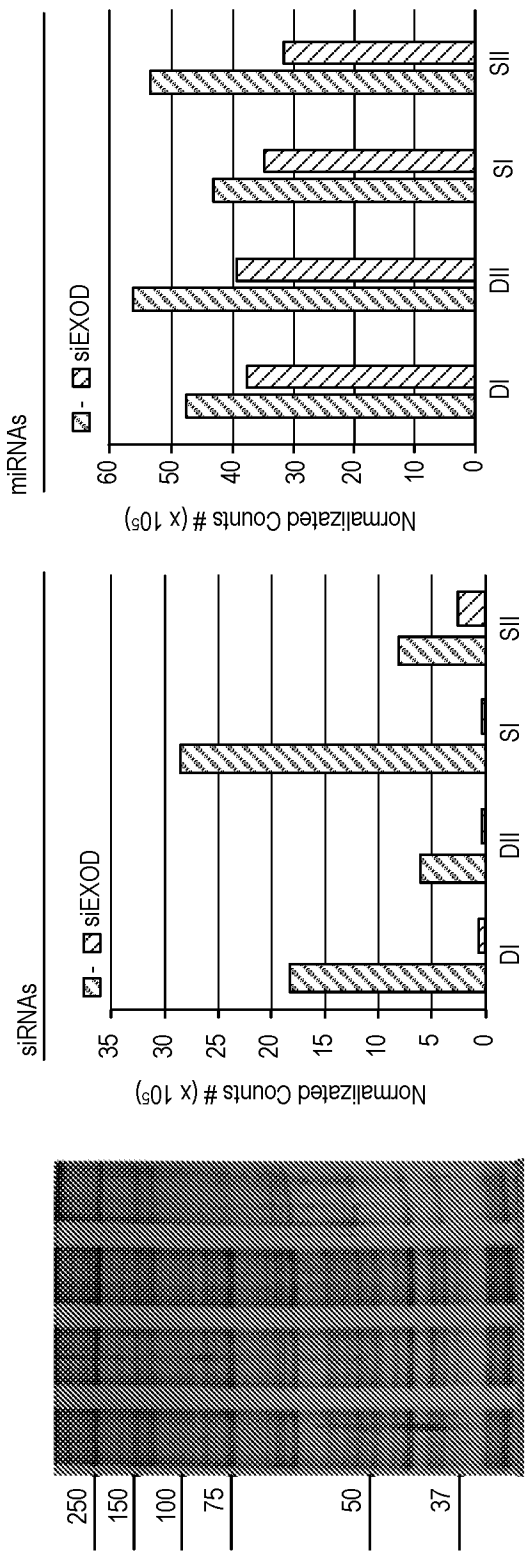

miRNAs
- siEXOD
Normalized Counts # (x 10⁵)
0
10
20
30
40
50
60
DI
DII
SI
SII
siRNAs
- siEXOD
Normalized Counts # (x 10⁵)
0
5
10
15
20
25
30
35
DI
DII
SI
SII
DICER IP
250
150
100
75
50
37

FIG. 20

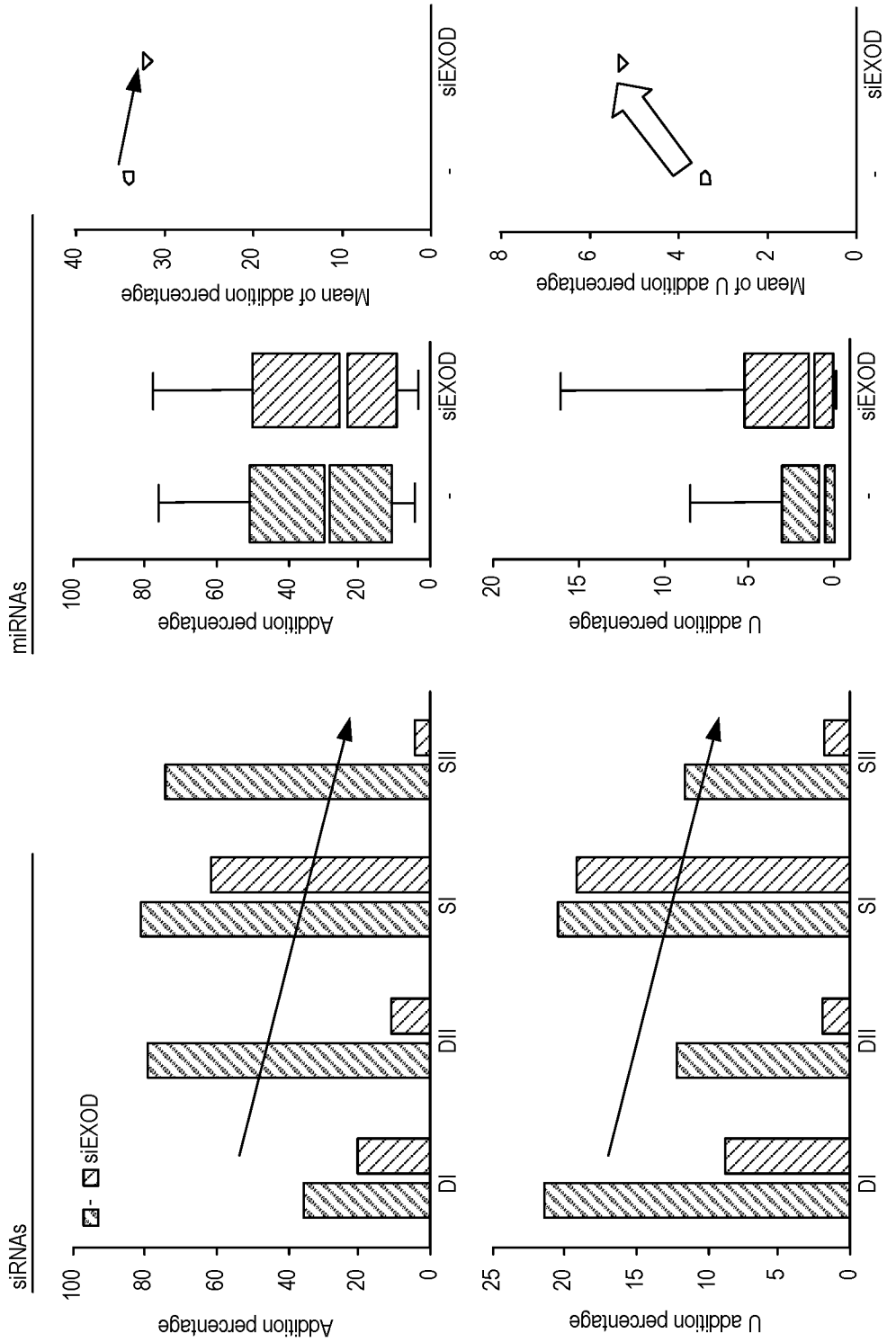

siRNAs
miRNAs
- siEXOD
Addition percentage
U addition percentage
Mean of addition percentage
Mean of U addition percentage
DI
DII
SI
SII
siEXOD
-

FIG. 21

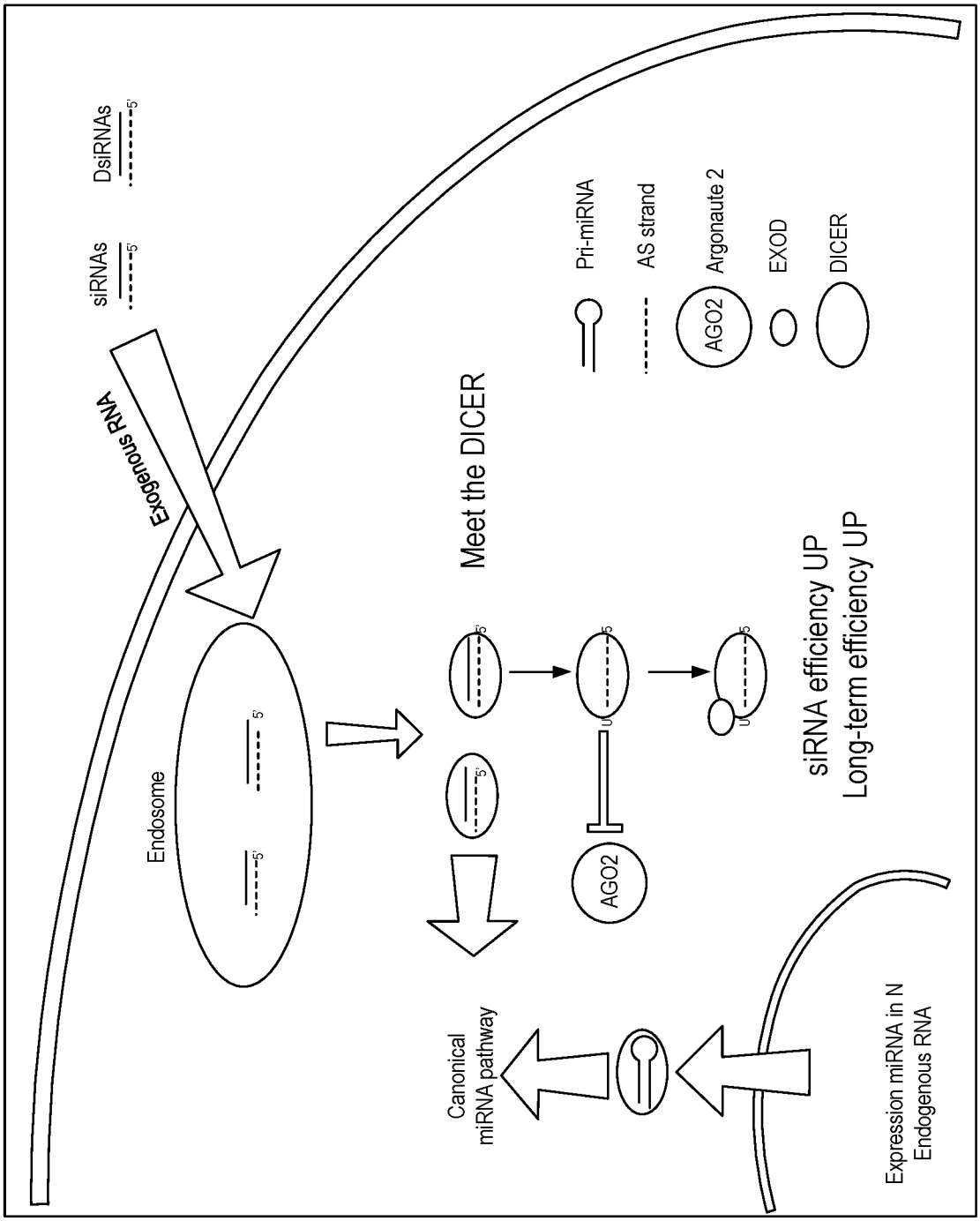

siRNAs
DsiRNAs
Exogenous RNA
Endosome
Meet the DICER
Pri-miRNA
AS strand
Argonaute 2
EXOD
DICER
AGO2
AGO2
siRNA efficiency UP
Long-term efficiency UP
Canonical
miRNA pathway
Expression miRNA in N
Endogenous RNA

FIG. 22

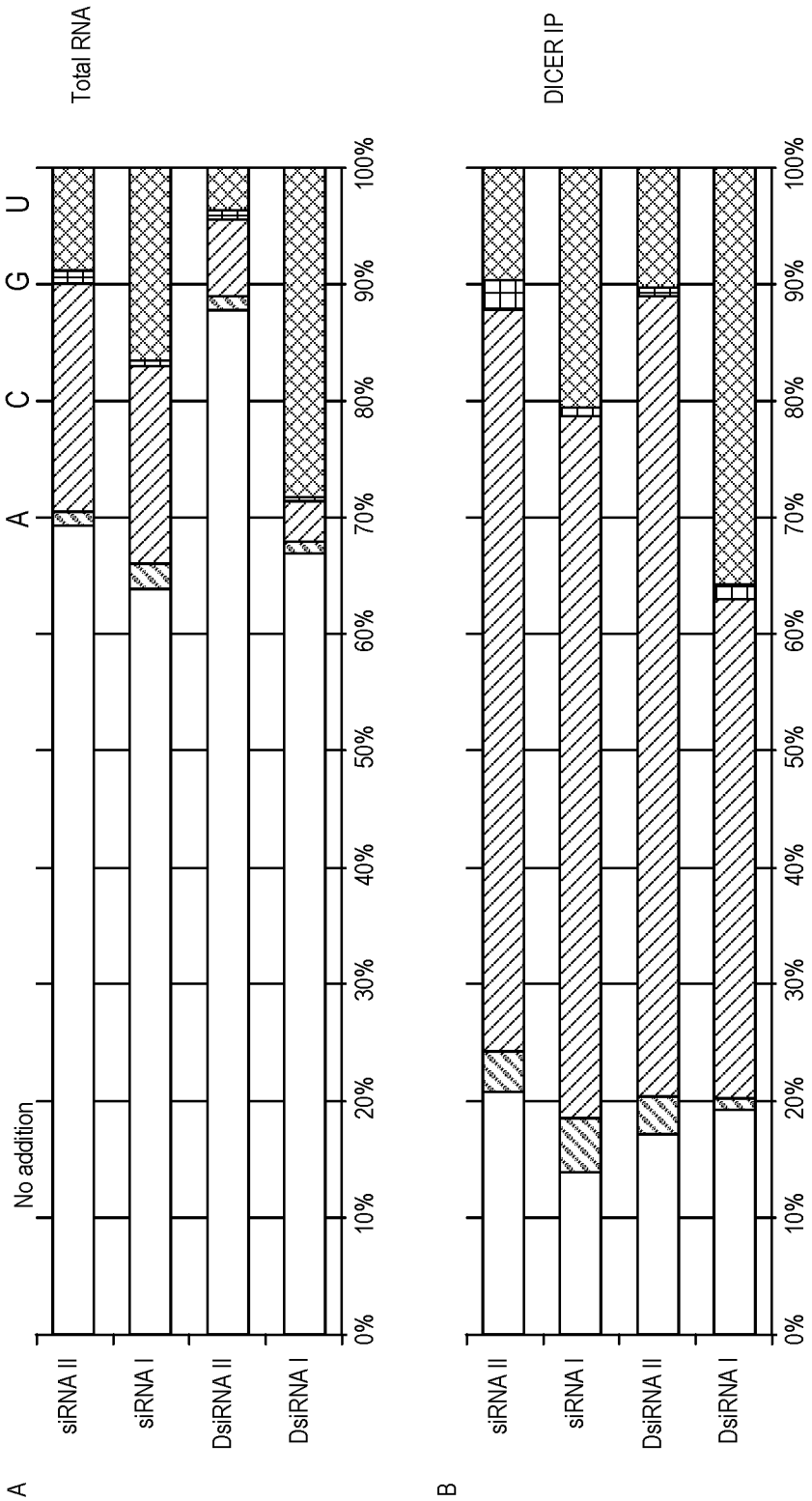

A
Total RNA
No addition
siRNA II
siRNA I
DsiRNA II
DsiRNA I
A C G U
0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
B
DICER IP
siRNA II
siRNA I
DsiRNA II
DsiRNA I
0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

FIG. 23

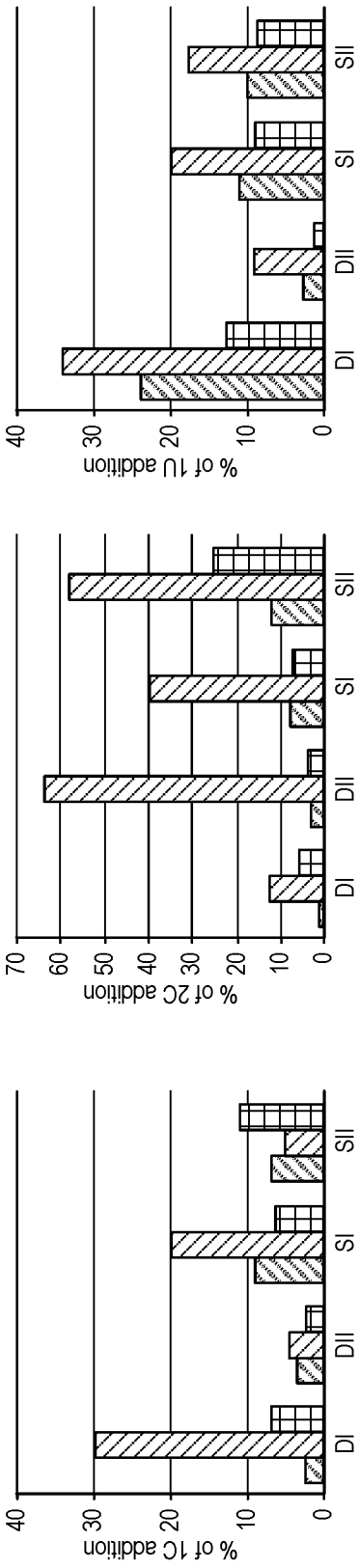
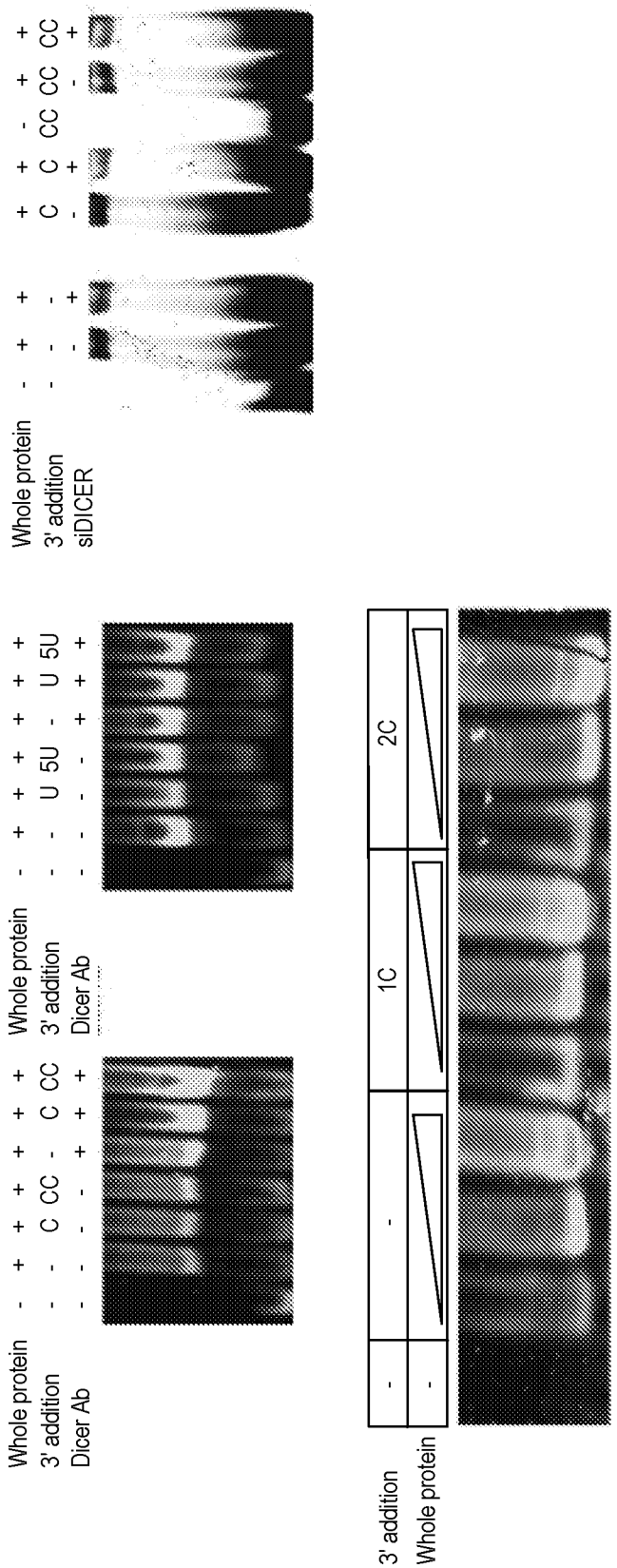

% of 1U addition
0
10
20
30
40
DI
DII
SI
SII
% of 2C addition
0
10
20
30
40
50
60
70
DI
DII
SI
SII
% of 1C addition
0
10
20
30
40
DI
DII
SI
SII

FIG. 24


Whole protein - + + +

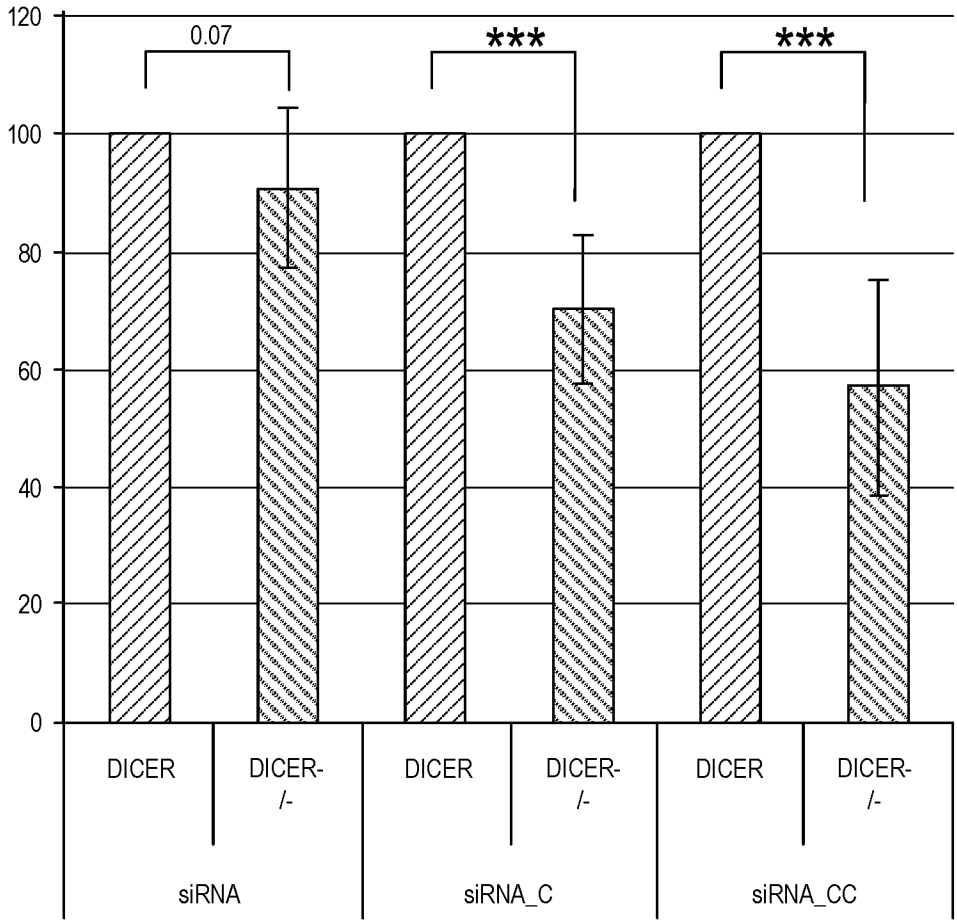

120
100
80
60
40
20
0
0.07
***
***
DICER
DICER-/-
DICER
DICER-/-
DICER
DICER-/-
siRNA
siRNA_C
siRNA_CC

FIG. 26

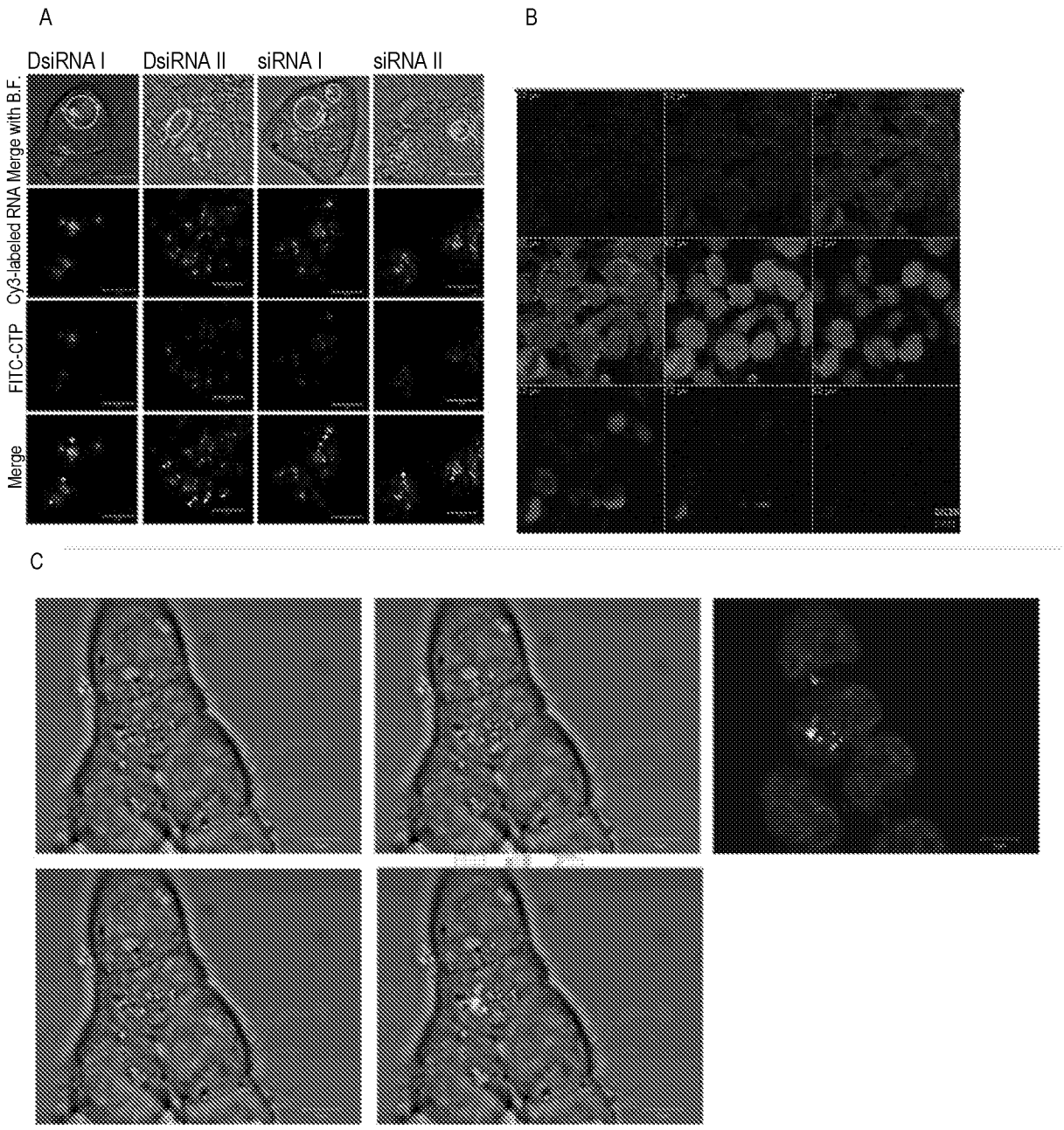

A
DsiRNA I
DsiRNA II
siRNA I
siRNA II
Cy3-labeled RNA Merge with B.F.
FITC-CTP
Merge
B
C

FIG. 27

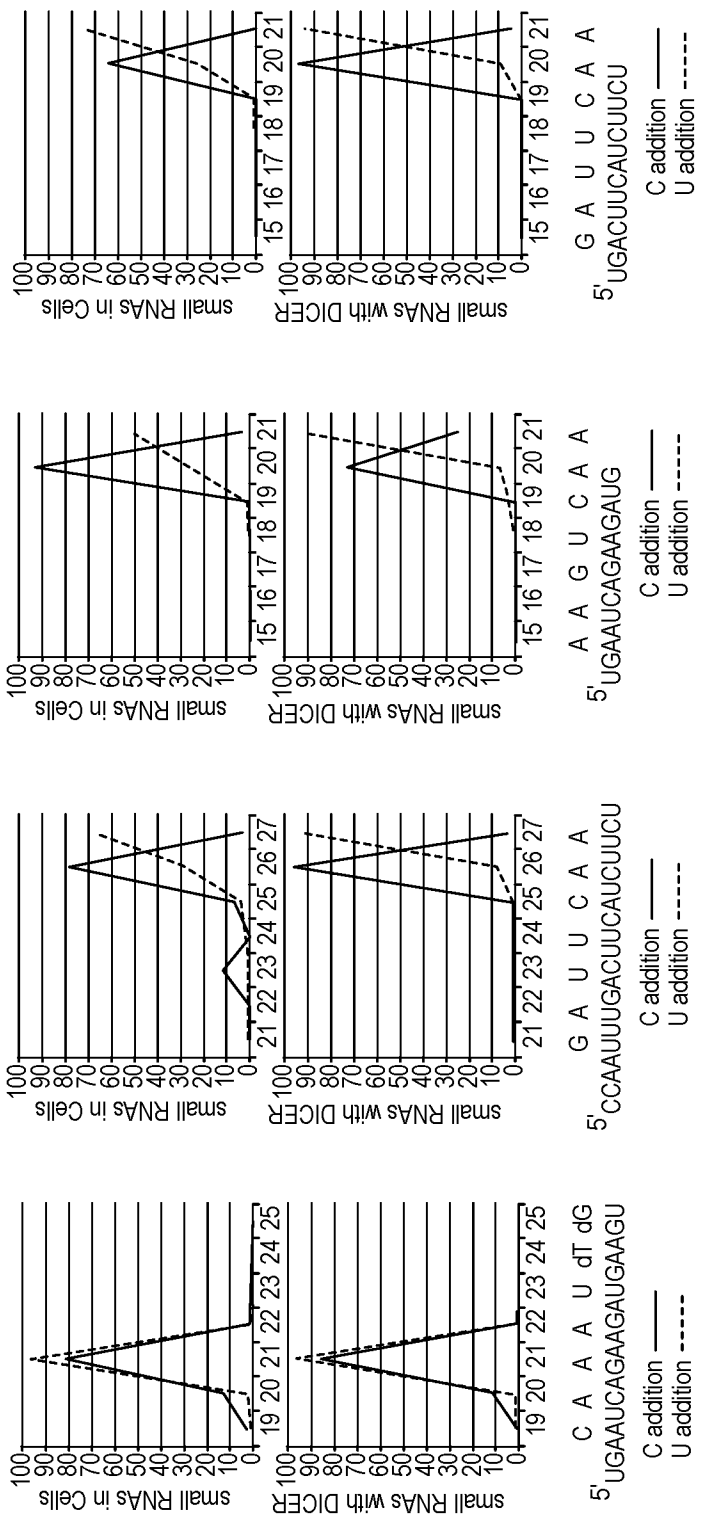

small RNAs in Cells
small RNAs with DICER
5'UGAAUCAGAAGAUGAAGU
C A A A U dT dG
5'CCAAUUUGACUUCAUCUUCU
G A U U C A A
5'UGAAUCAGAAGAUG
A A G U C A A
5'UGACUUCAUCUUCU
G A U U C A A
C addition
U addition

FIG. 28

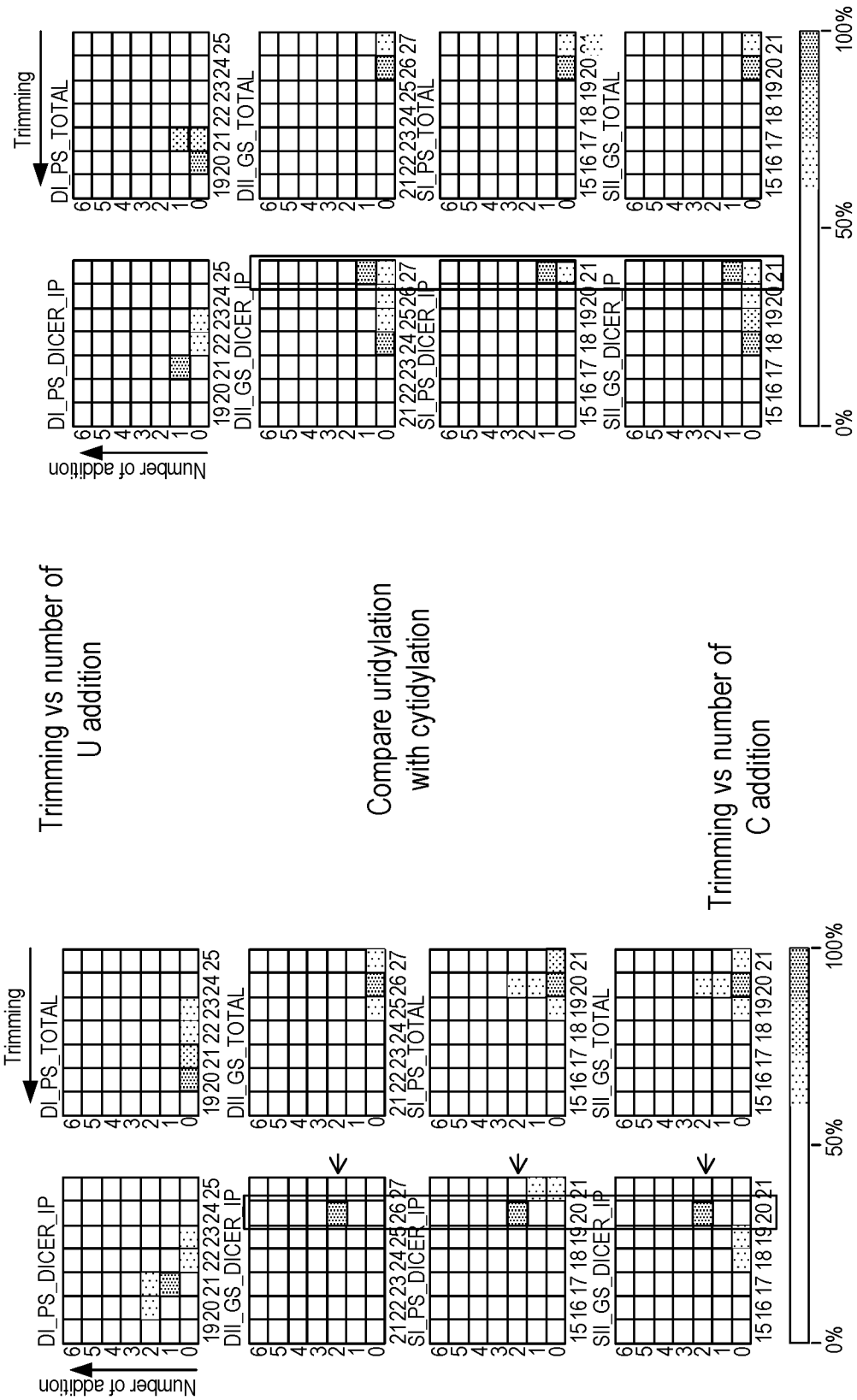

Trimming vs number of U addition
Compare uridylation with cytidylation
Trimming vs number of C addition
Trimming
Number of addition
DI_PS_DICER_IP
DI_PS_TOTAL
DII_GS_DICER_IP
DII_GS_TOTAL
SI_PS_DICER_IP
SI_PS_TOTAL
SII_GS_DICER_IP
SII_GS_TOTAL
0%
50%
100%

FIG. 29

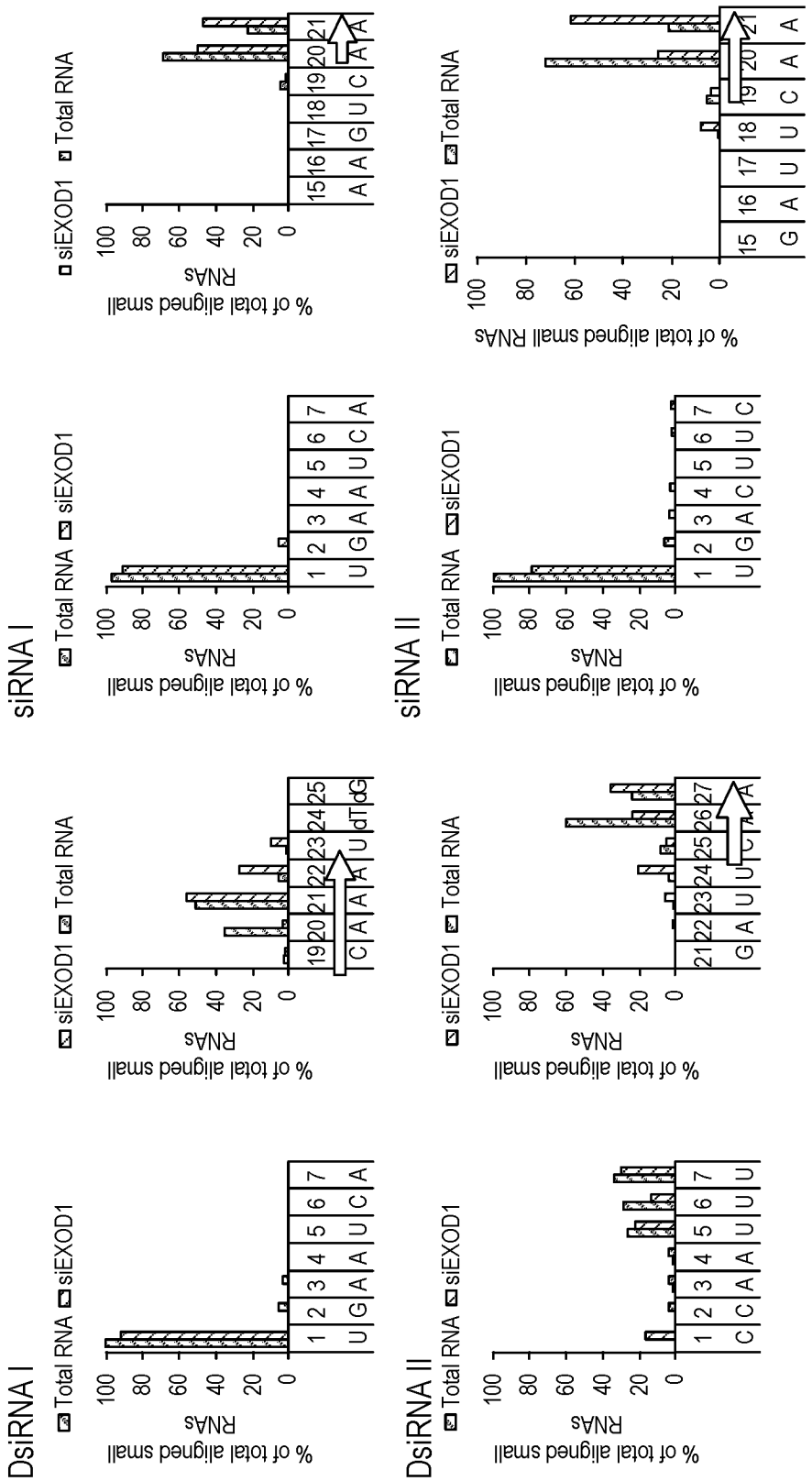
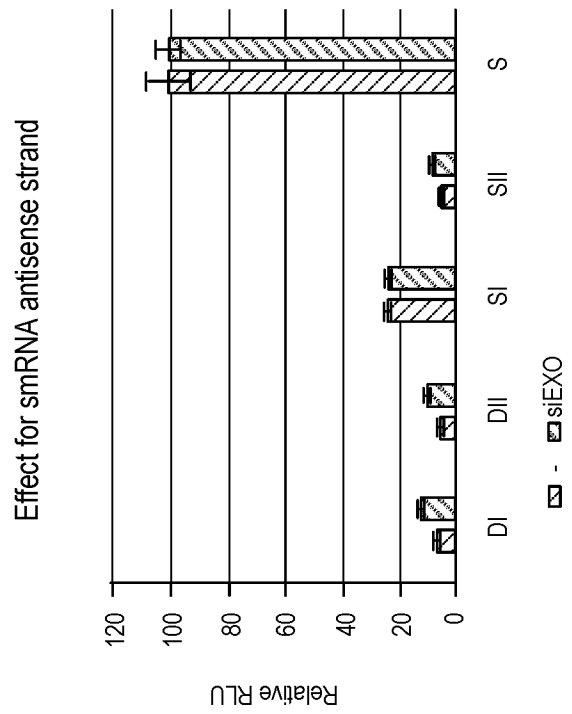
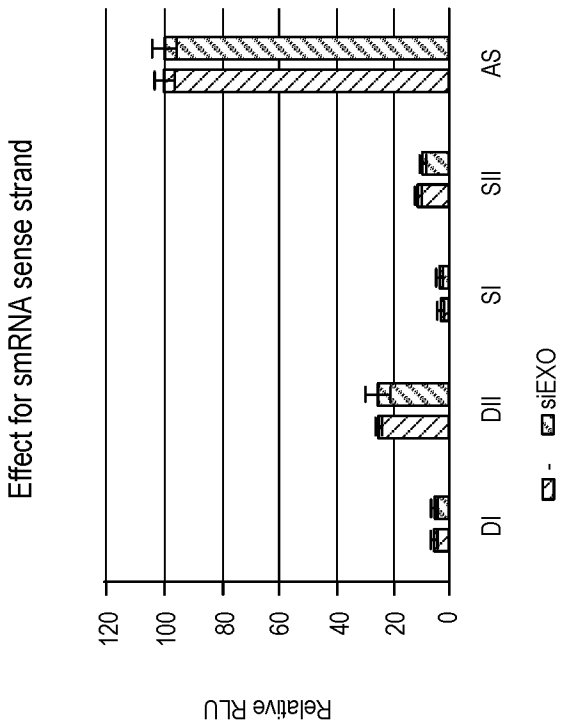

DsiRNA I
DsiRNA II
siRNA I
siRNA II
Total RNA
siEXOD1
% of total aligned small RNAs

FIG. 30





t-test	S	AS
		no vs siEXOD1
DI	0.00002	0.27621
DII	0.00004	0.89390
SI	0.45746	0.30997
SII	0.00013	0.00496

FIG. 31

Figure 32

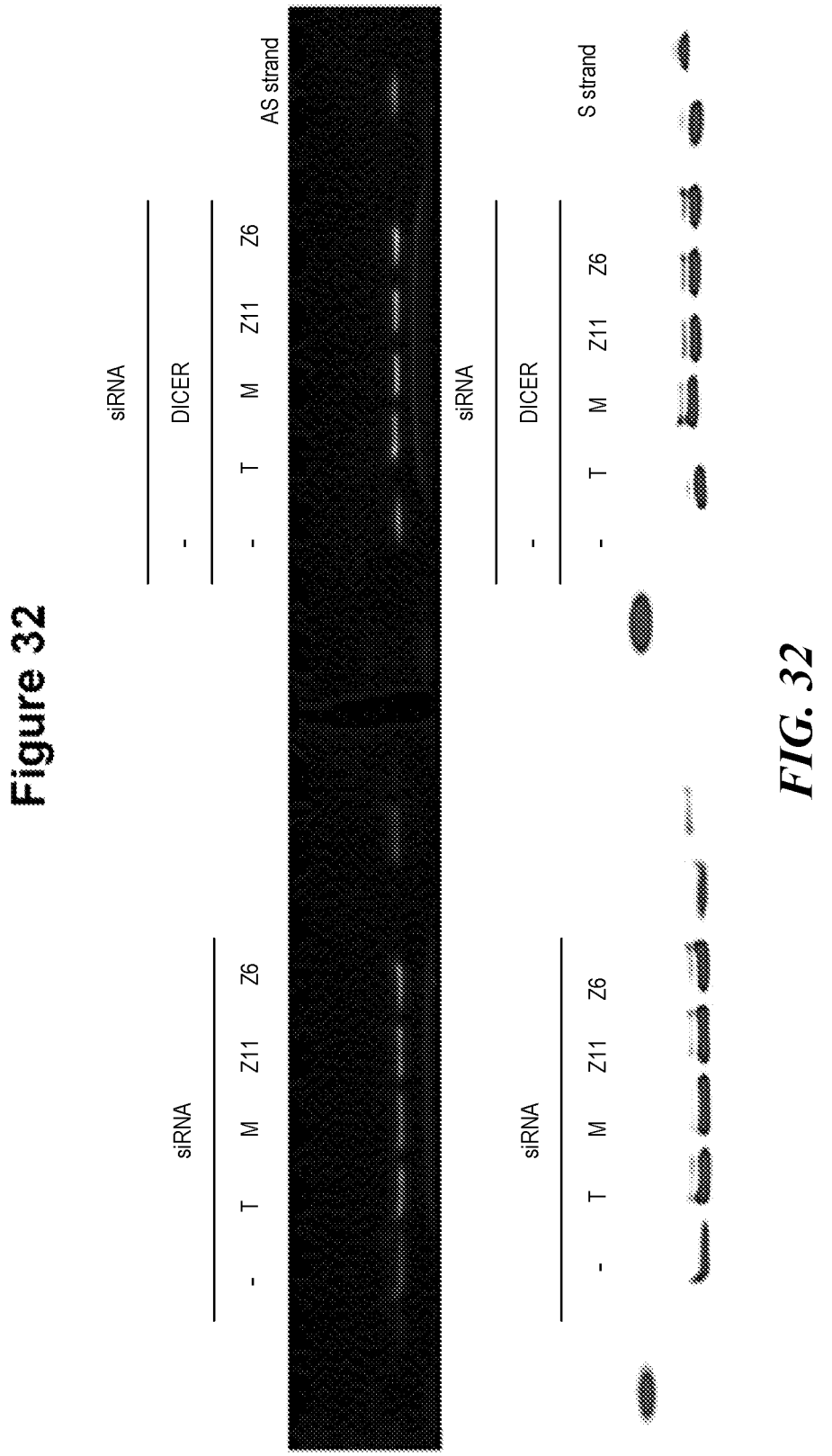


FIG. 32

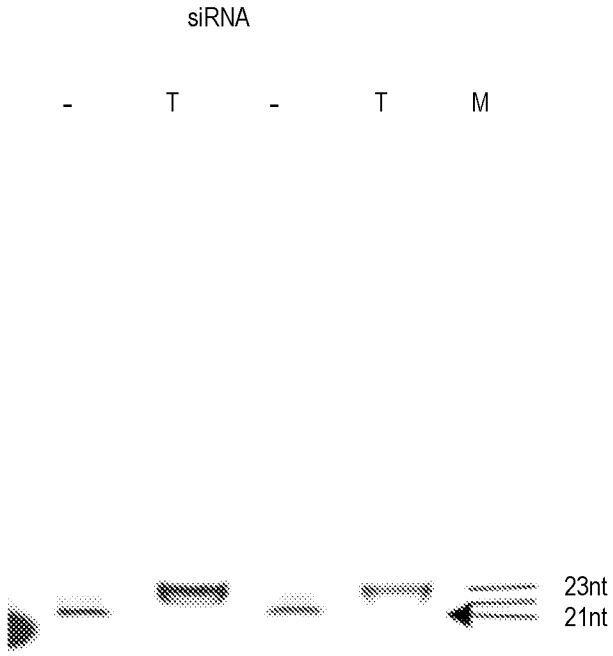


FIG. 33

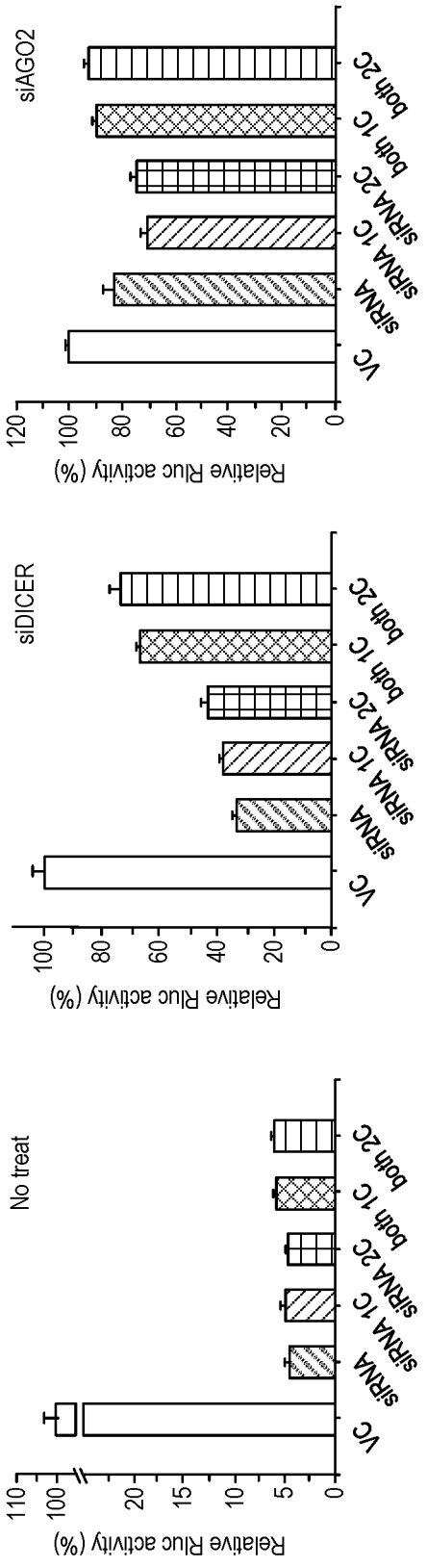
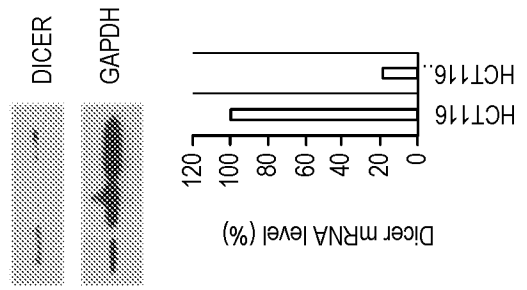

No treat
siDICER
siAGO2
Relative Rluc activity (%)
VC
siRNA
siRNA 1C
siRNA 2C
both 1C
both 2C

FIG. 34


DICER
GAPDH
HCT116
HCT116
Dicer mRNA level (%)
0
20
40
60
80
100
120

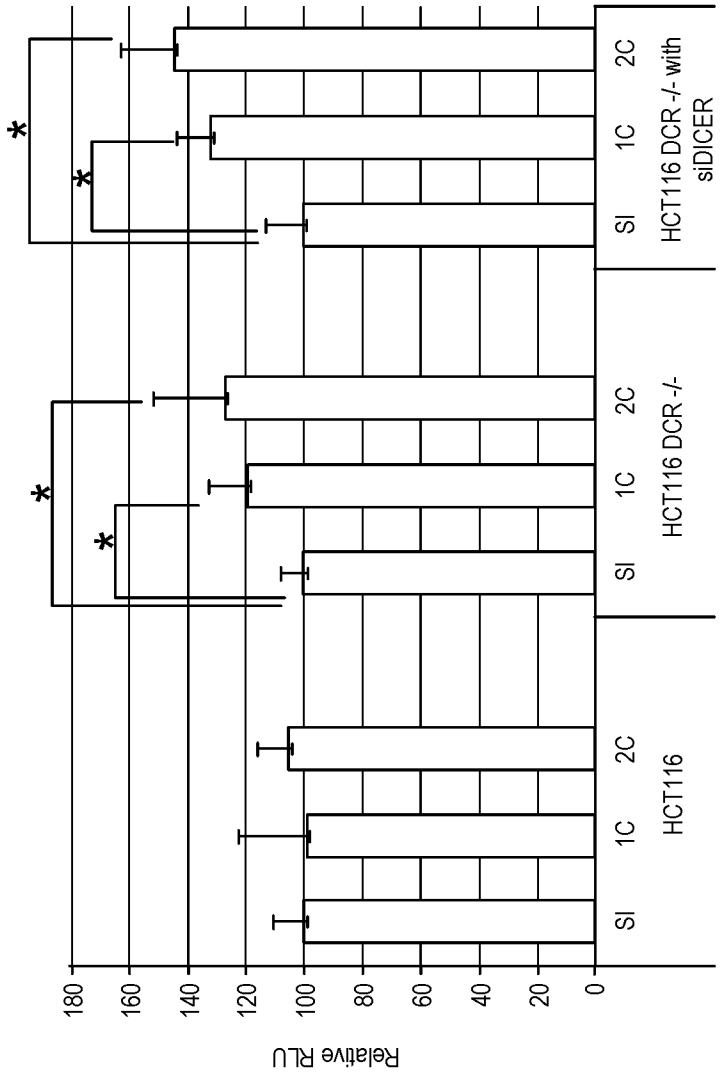

Relative RLU
0
20
40
60
80
100
120
140
160
180
SI
1C
2C
HCT116
HCT116 DCR -/-
HCT116 DCR -/- with siDICER
*
*
*
*

FIG. 35

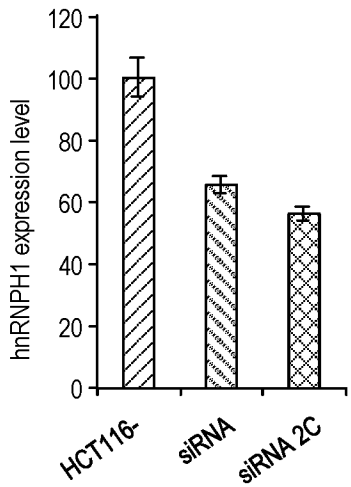
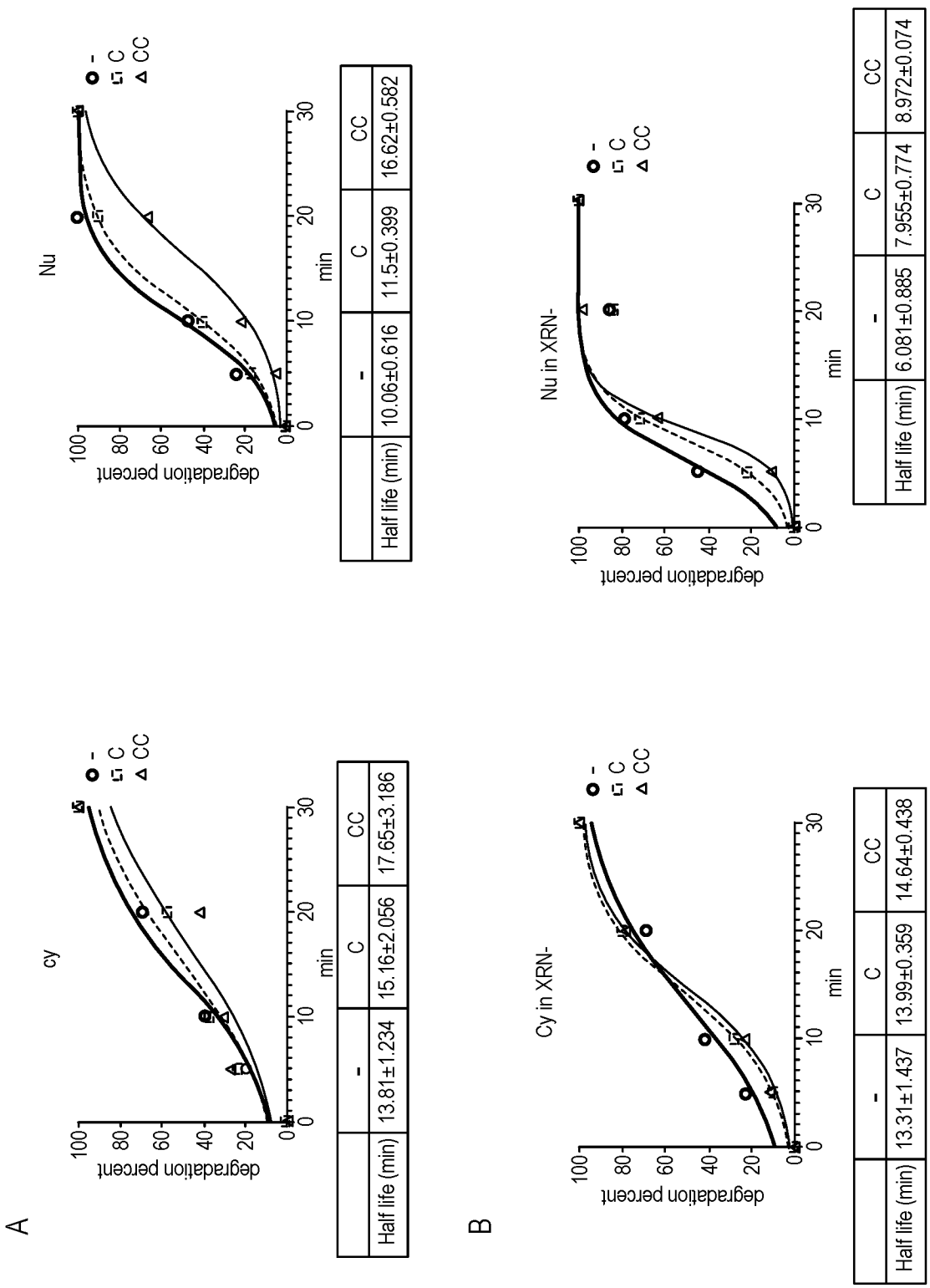

120
100
80
60
40
20
0
hnRNPH1 expression level
HCT116-
siRNA
siRNA 2C

FIG. 36



	-	C	CC
Half life (min)	13.81±1.234	15.16±2.056	17.65±3.186

	-	C	CC
Half life (min)	10.06±0.616	11.5±0.399	16.62±0.582

	-	C	CC
Half life (min)	13.31±1.437	13.99±0.359	14.64±0.438

	-	C	CC
Half life (min)	6.081±0.885	7.955±0.774	8.972±0.074

FIG. 37

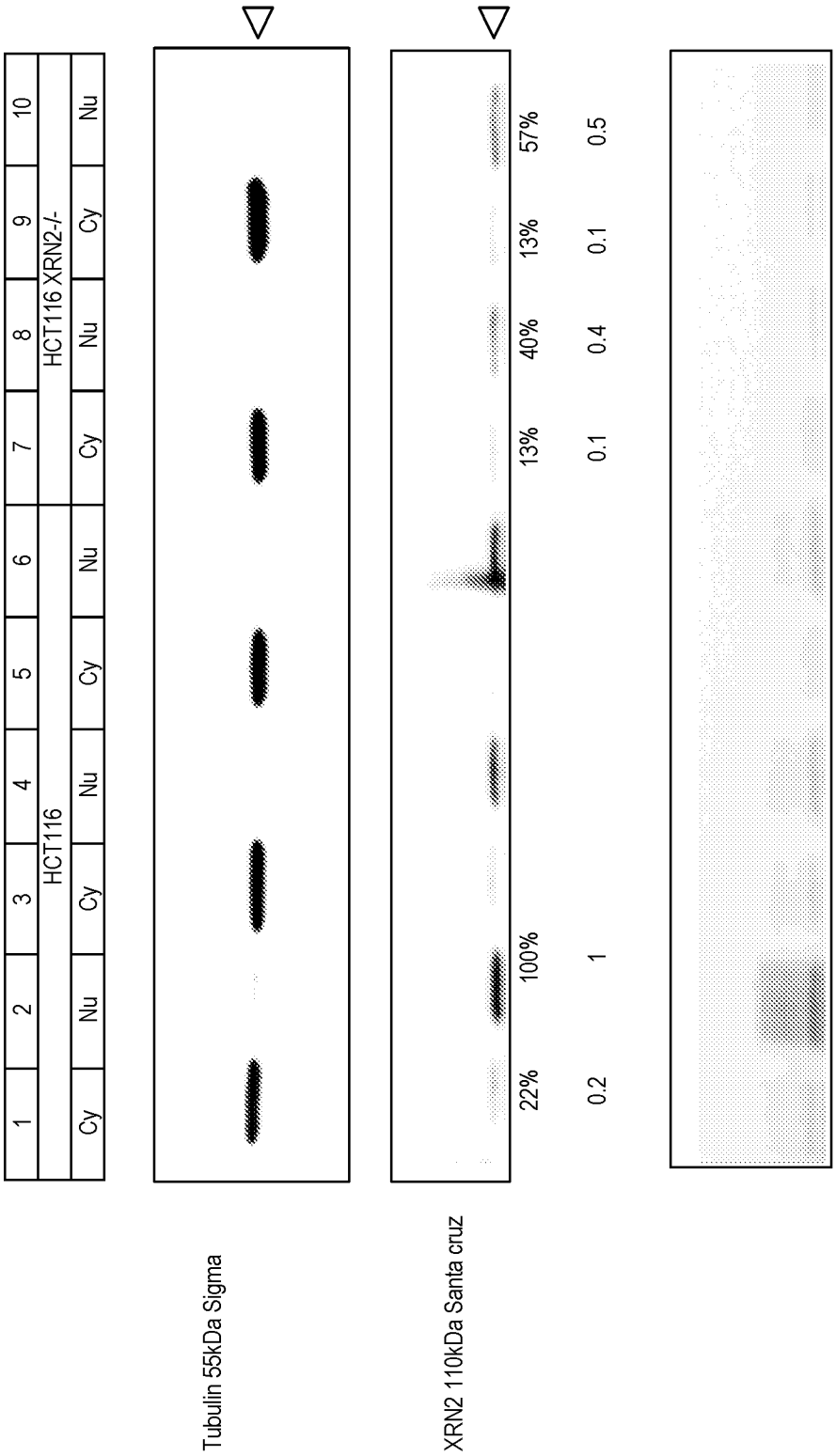

1
2
3
4
5
6
7
8
9
10
HCT116
HCT116 XRN2-/-
Cy
Nu
Cy
Nu
Cy
Nu
Cy
Nu
Cy
Nu
Tubulin 55kDa Sigma
XRN2 110kDa Santa cruz
22%
100%
13%
40%
13%
57%
0.2
1
0.1
0.4
0.1
0.5

FIG. 38

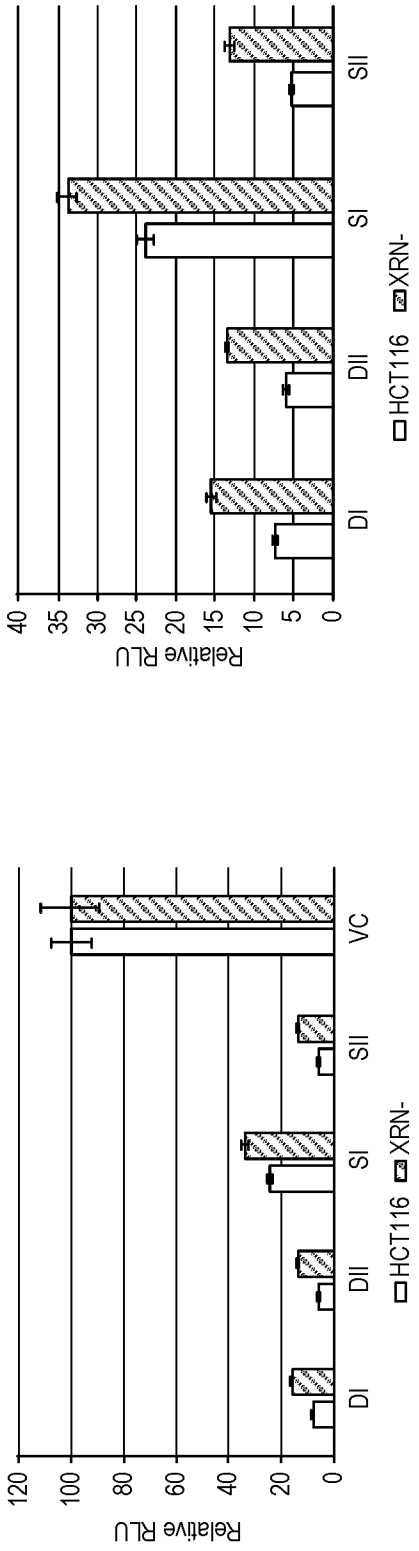

Relative RLU
0
20
40
60
80
100
120
DI
DII
SI
SII
VC
HCT116
XRN-
Relative RLU
0
5
10
15
20
25
30
35
40
DI
DII
SI
SII
HCT116
XRN-

FIG. 39

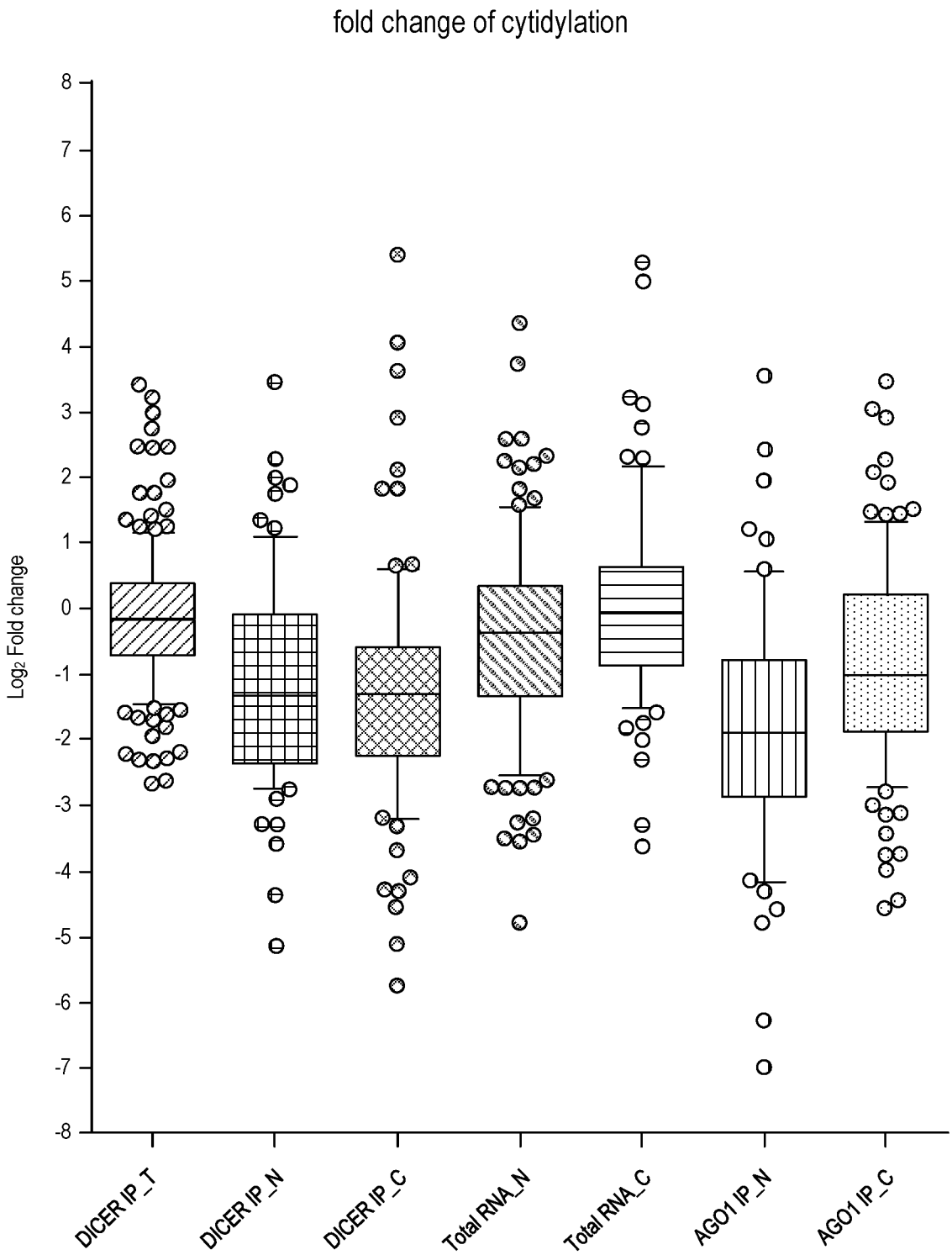

fold change of cytidylation
Log2 Fold change
8
7
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
-8
DICER IP_T
DICER IP_N
DICER IP_C
Total RNA_N
Total RNA_C
AGO1 IP_N
AGO1 IP_C

FIG. 40

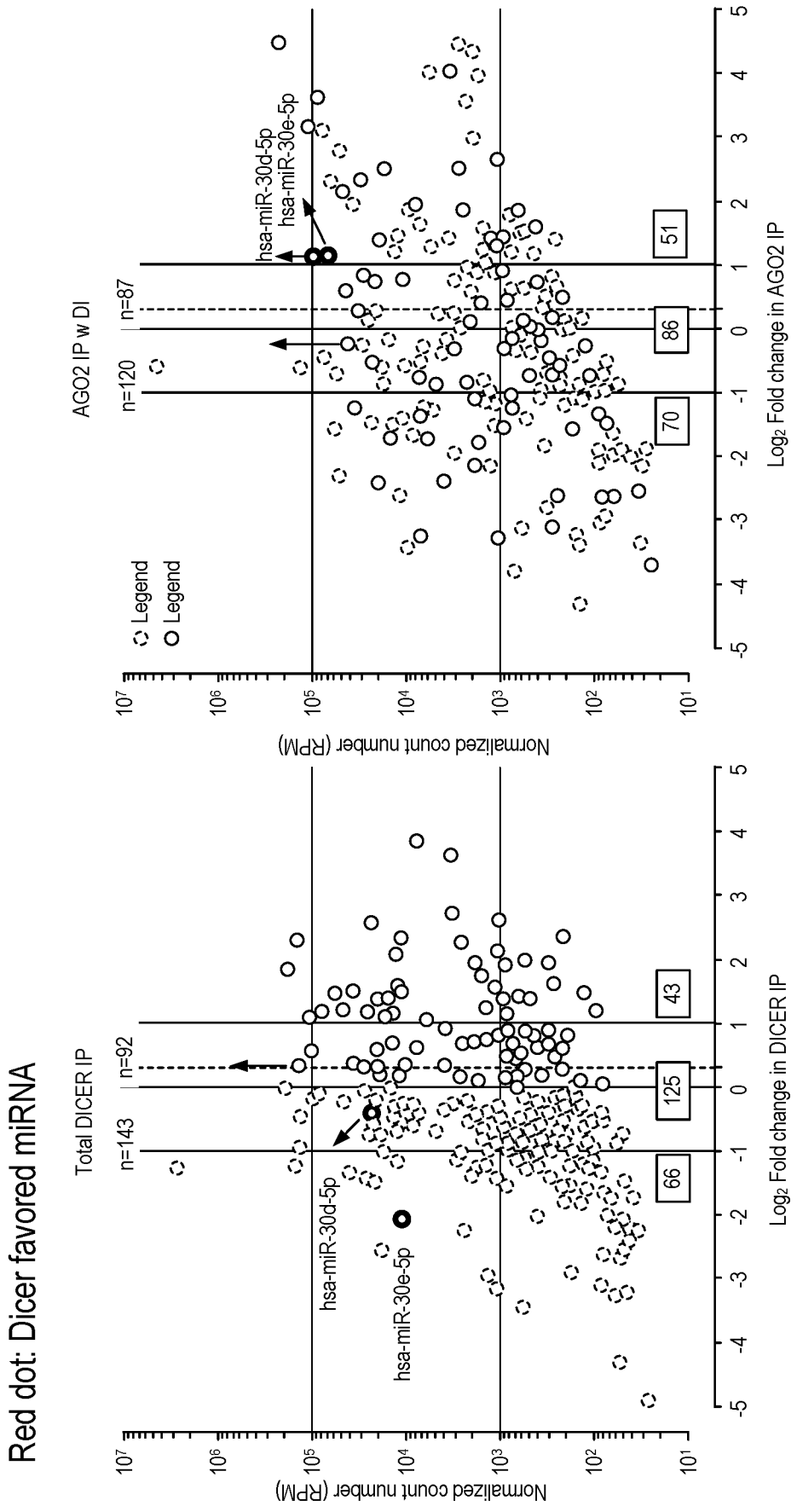

Red dot: Dicer favored miRNA
Total DICER IP
n=143
n=92
hsa-miR-30d-5p
hsa-miR-30e-5p
66
125
43
Normalized count number (RPM)
Log2 Fold change in DICER IP
AGO2 IP w DI
n=120
n=87
Legend
Legend
hsa-miR-30d-5p
hsa-miR-30e-5p
70
86
51
Normalized count number (RPM)
Log2 Fold change in AGO2 IP

FIG. 41

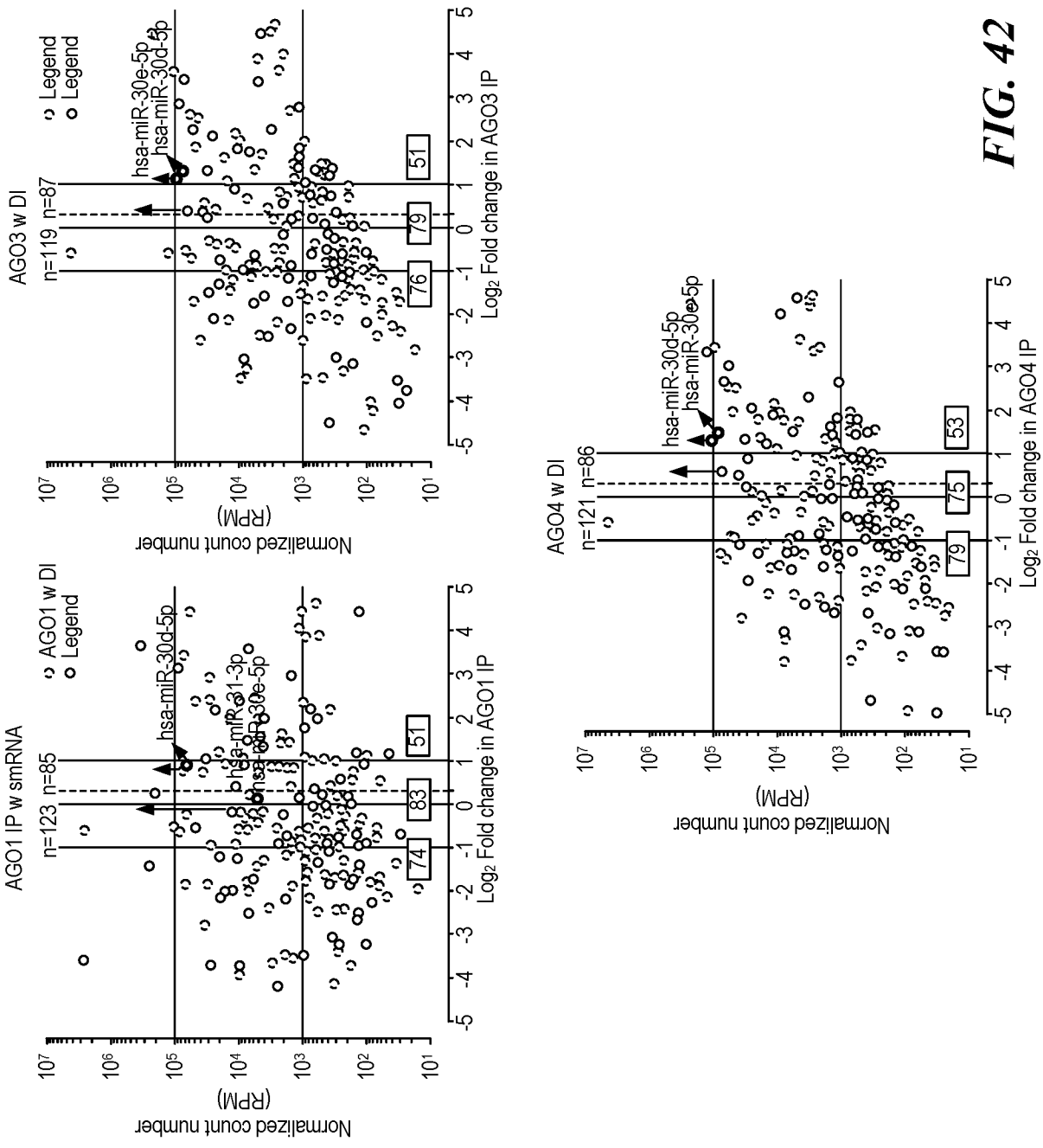

AGO1 IP w smRNA
AGO1 w DI
Legend
n=123 n=85
hsa-miR-30d-5p
hsa-miR-31-3p
hsa-miR-30e-5p
74
83
51
Log2 Fold change in AGO1 IP
Normalized count number (RPM)
AGO3 w DI
Legend
n=119 n=87
hsa-miR-30e-5p
hsa-miR-30d-5p
76
79
51
Log2 Fold change in AGO3 IP
Normalized count number (RPM)
AGO4 w DI
n=121 n=86
hsa-miR-30d-5p
hsa-miR-30e-5p
79
75
53
Log2 Fold change in AGO4 IP
Normalized count number (RPM)

FIG. 42

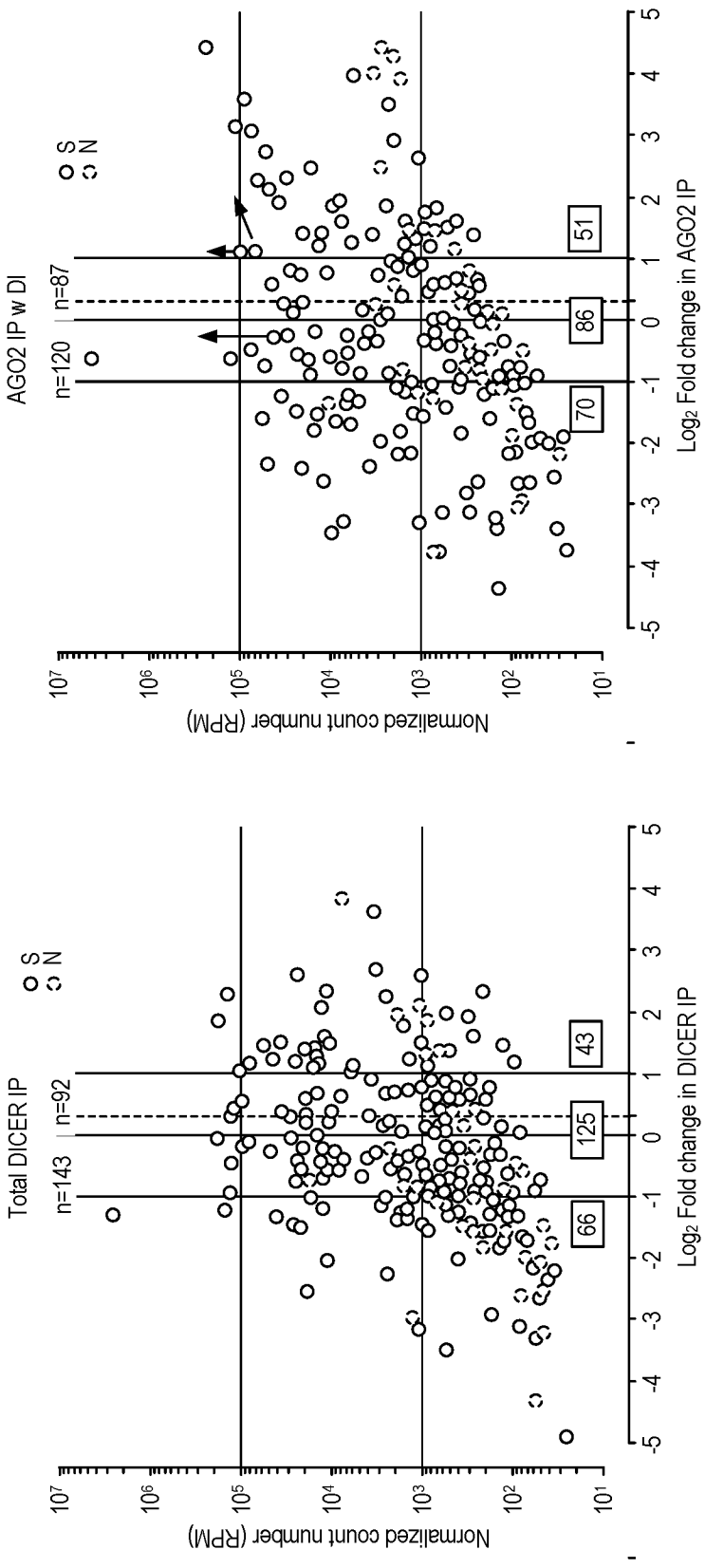

AGO2 IP w DI
n=120
n=87
S
N
51
86
70
Normalized count number (RPM)
Log2 Fold change in AGO2 IP
Total DICER IP
n=143
n=92
S
N
43
125
66
Normalized count number (RPM)
Log2 Fold change in DICER IP

FIG. 43

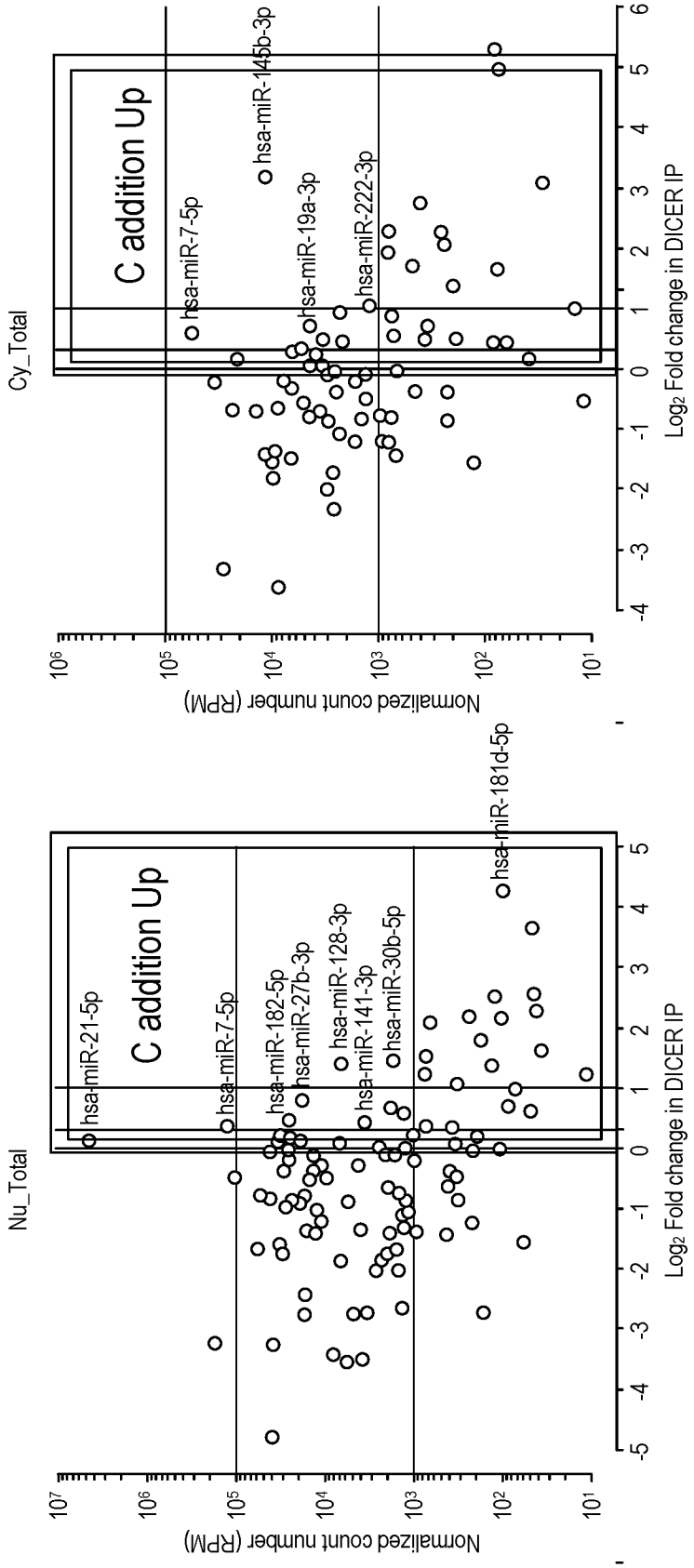

Cy_Total
C addition Up
hsa-miR-7-5p
hsa-miR-19a-3p
hsa-miR-222-3p
hsa-miR-145b-3p
Normalized count number (RPM)
Log2 Fold change in DICER IP
Nu_Total
C addition Up
hsa-miR-21-5p
hsa-miR-7-5p
hsa-miR-182-5p
hsa-miR-27b-3p
hsa-miR-128-3p
hsa-miR-141-3p
hsa-miR-30b-5p
hsa-miR-181d-5p
Normalized count number (RPM)
Log2 Fold change in DICER IP

FIG. 44

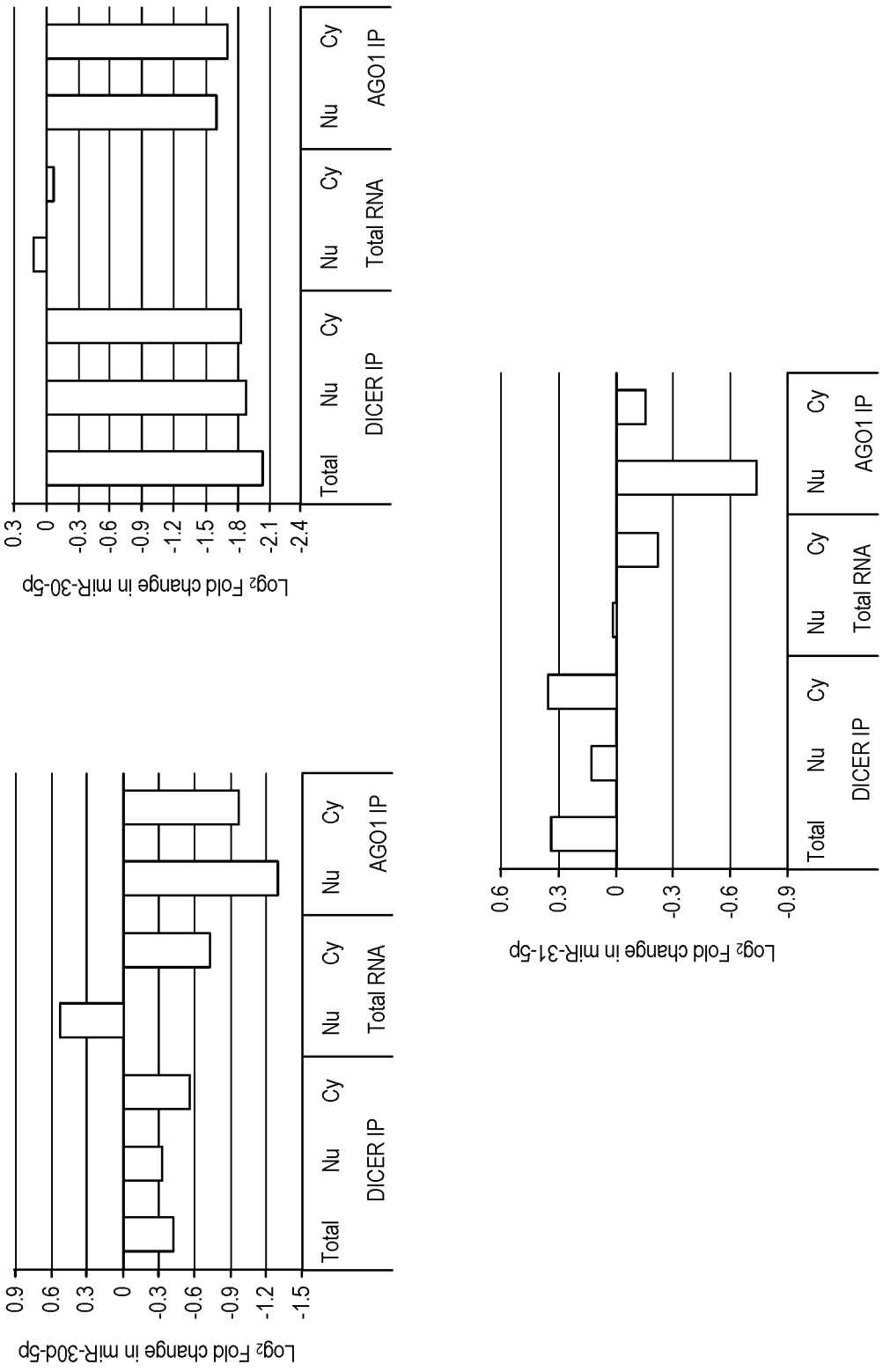

Log2 Fold change in miR-30d-5p
0.9
0.6
0.3
0
-0.3
-0.6
-0.9
-1.2
-1.5
Total
Nu
Cy
DICER IP
Nu
Cy
Total RNA
Nu
Cy
AGO1 IP
Log2 Fold change in miR-30-5p
0.3
0
-0.3
-0.6
-0.9
-1.2
-1.5
-1.8
-2.1
-2.4
Total
Nu
Cy
DICER IP
Nu
Cy
Total RNA
Nu
Cy
AGO1 IP
Log2 Fold change in miR-31-5p
0.6
0.3
0
-0.3
-0.6
-0.9
Total
Nu
Cy
DICER IP
Nu
Cy
Total RNA
Nu
Cy
AGO1 IP

FIG. 45

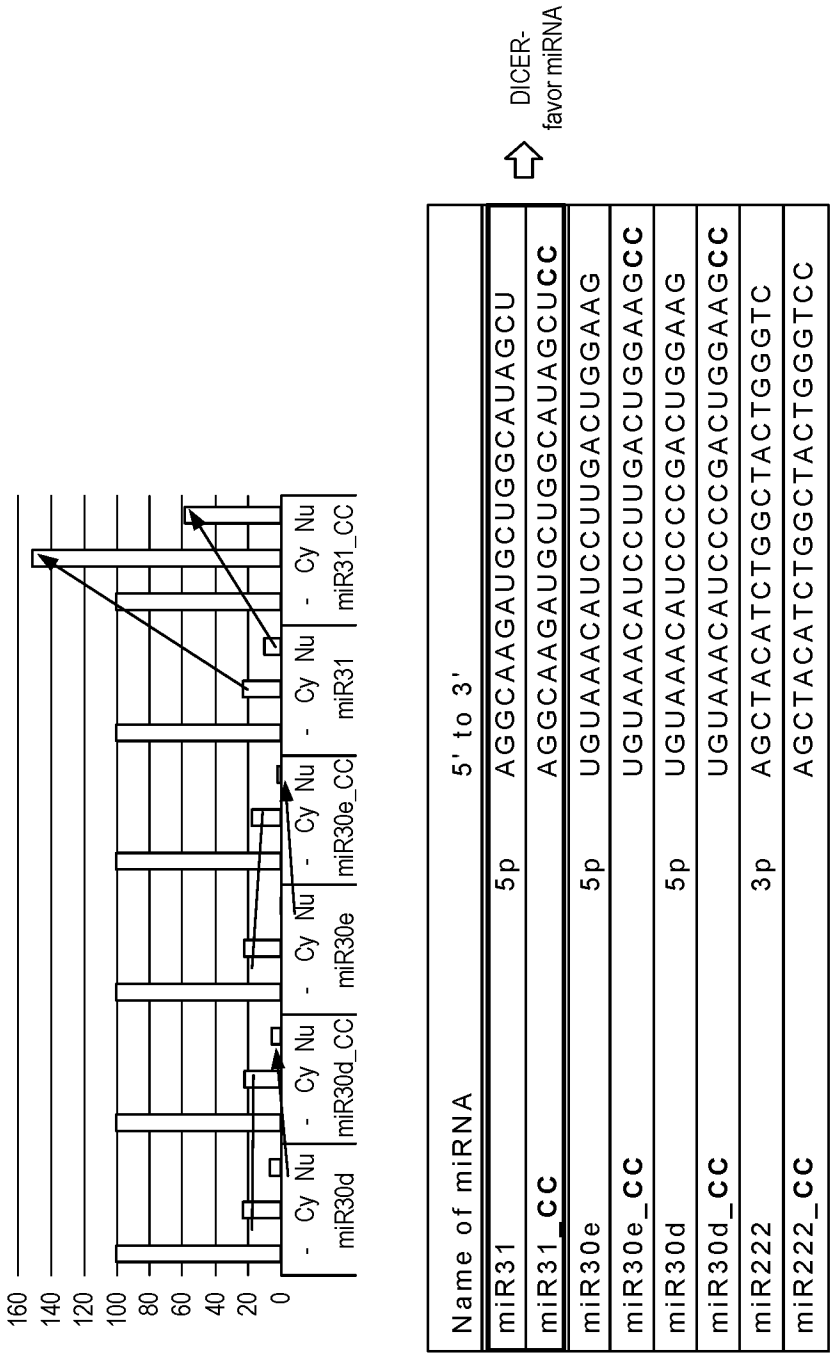

160
140
120
100
80
60
40
20
0
- Cy Nu miR30d
- Cy Nu miR30d_CC
- Cy Nu miR30e
- Cy Nu miR30e_CC
- Cy Nu miR31
- Cy Nu miR31_CC
DICER-favor miRNA
Name of miRNA
5' to 3'
miR31 5p AGGCAAGAUGCUGGCAUAGCU
miR31_CC AGGCAAGAUGCUGGCAUAGCUCC
miR30e 5p UGUAAACAUCCUUGACUGGAAG
miR30e_CC UGUAAACAUCCUUGACUGGAAGCC
miR30d 5p UGUAAACAUCCCCGACUGGAAG
miR30d_CC UGUAAACAUCCCCGACUGGAAGCC
miR222 3p AGCTACATCTGGCTACTGGGTC
miR222_CC AGCTACATCTGGCTACTGGGTCC

FIG. 46

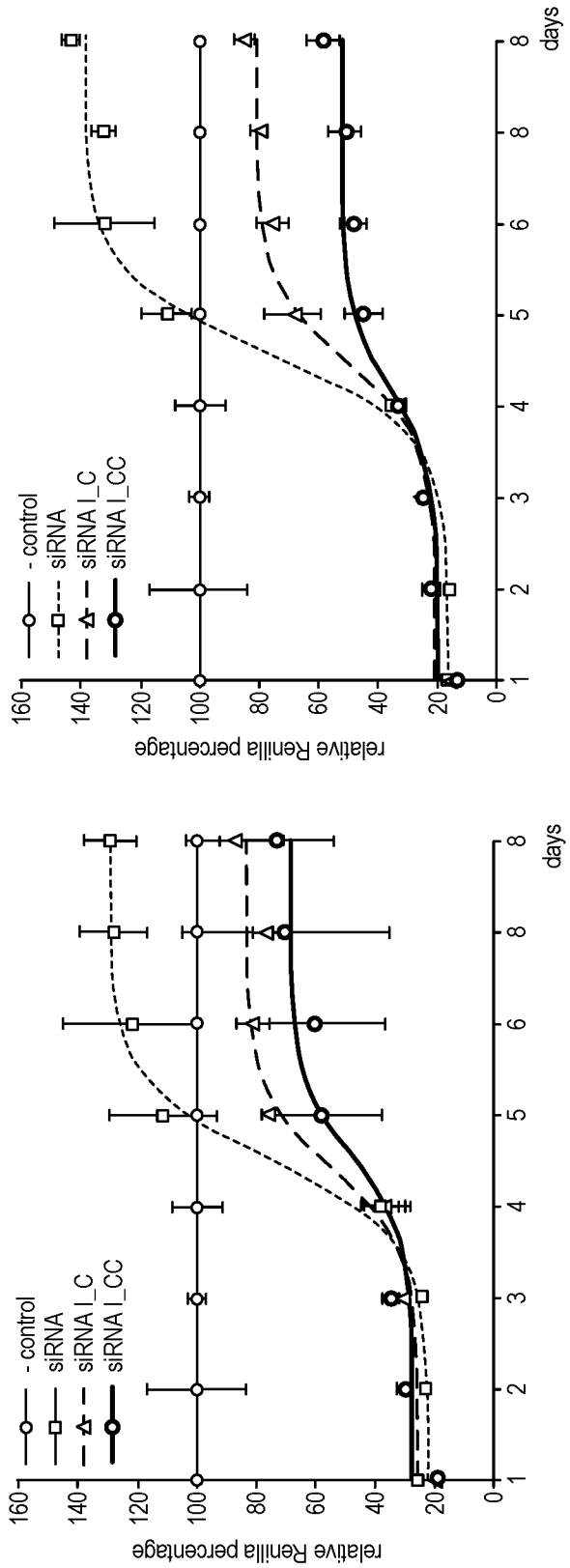

relative Renilla percentage
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
8
days
- control
siRNA
siRNA I_C
siRNA I_CC
2nM
smRNA
10nM
smRNA

FIG. 47

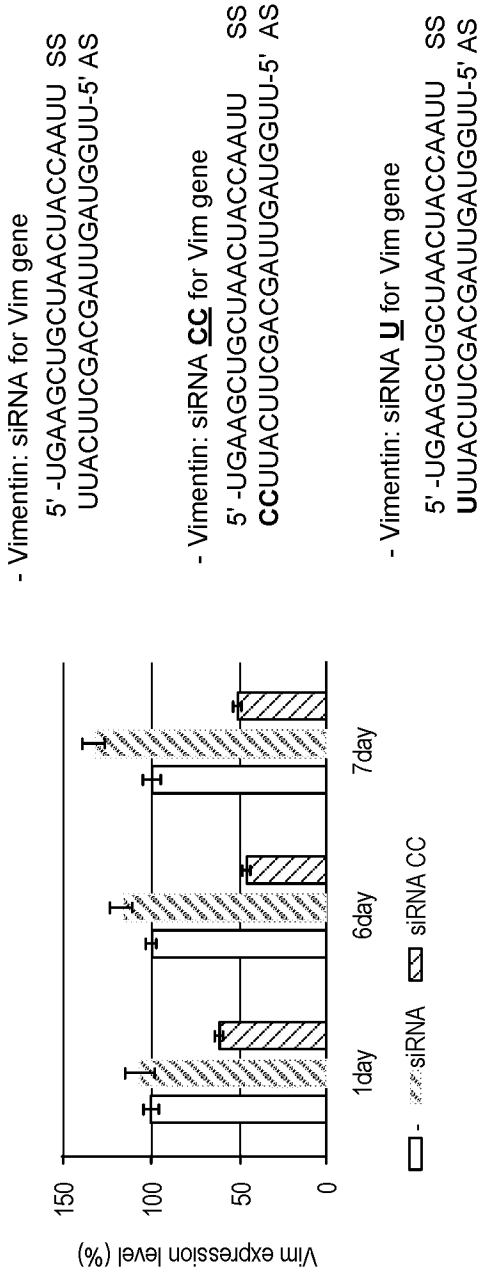

- Vimentin: siRNA for Vim gene
5' -UGAAGCUGCUAACUACCAAUU SS
UUACUUCGACGAUUGAUGGUU-5' AS
- Vimentin: siRNA CC for Vim gene
5' -UGAAGCUGCUAACUACCAAUU SS
CCUUACUUCGACGAUUGAUGGUU-5' AS
- Vimentin: siRNA U for Vim gene
5' -UGAAGCUGCUAACUACCAAUU SS
UUUACUUCGACGAUUGAUGGUU-5' AS
Vim expression level (%)
150
100
50
0
1day
6day
7day
- siRNA siRNA CC

FIG. 48

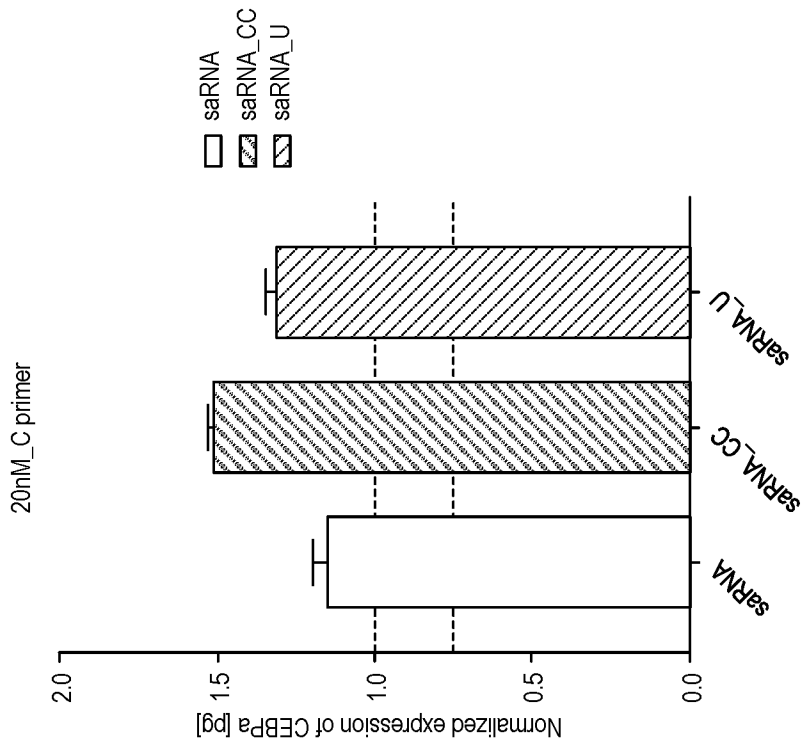

saRNA
saRNA_CC
saRNA_U
20nM_C primer
Normalized expression of CEBPa [pg]
2.0
1.5
1.0
0.5
0.0
saRNA
saRNA_CC
saRNA_U

FIG. 49

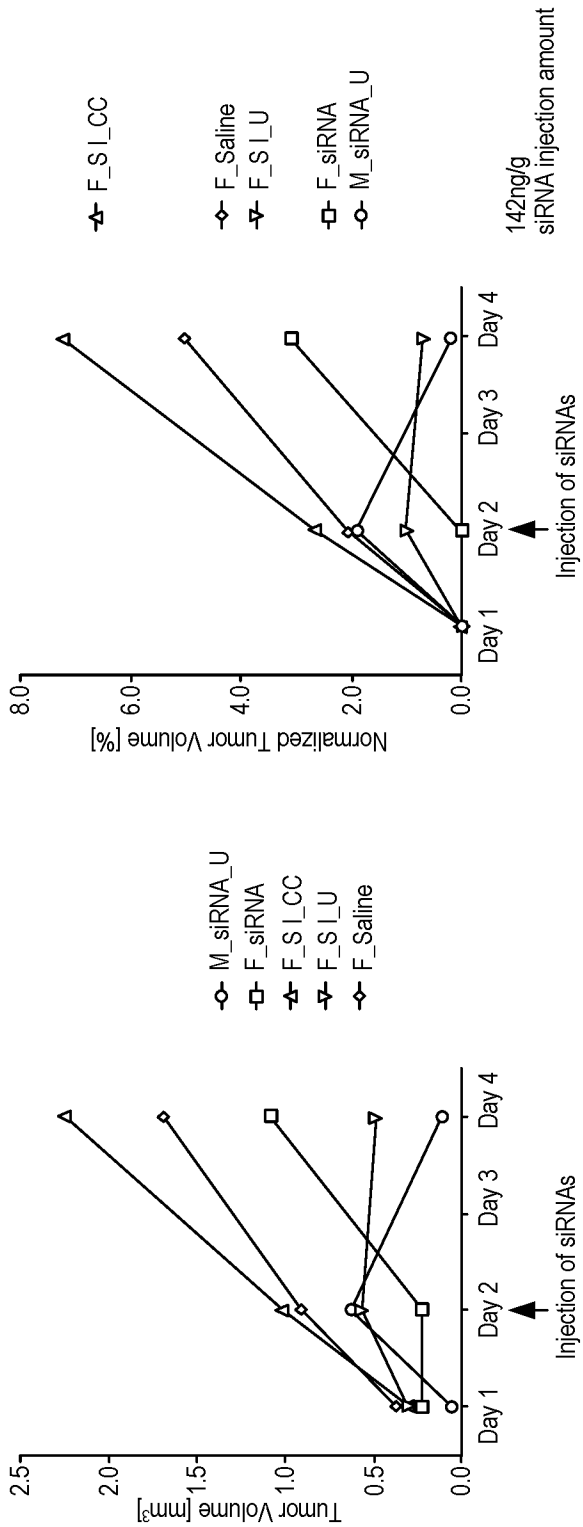

F_S I_CC
F_Saline
F_S I_U
F_siRNA
M_siRNA_U
142ng/g
siRNA injection amount
Normalized Tumor Volume [%]
8.0
6.0
4.0
2.0
0.0
Day 1
Day 2
Day 3
Day 4
Injection of siRNAs
M_siRNA_U
F_siRNA
F_S I_CC
F_S I_U
F_Saline
Tumor Volume [mm3]
2.5
2.0
1.5
1.0
0.5
0.0
Day 1
Day 2
Day 3
Day 4
Injection of siRNAs

FIG. 50

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/39622

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/39622

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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. ☐ Claims Nos.:
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:

3. ☒ Claims Nos.: 4-9, 14-19
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. 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 additional fees, this Authority did not invite payment of additional fees.

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 and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/39622

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12Q 1/68; C12N 15/113, 15/09; C12P 19/34 (2019.01)

CPC - C12Q 1/68; C12N 15/113, 15/09; C12P 19/34

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X –	(MUNOZ-TELLO, P et al.) Polyuridylation in Eukaryotes: A 3'-End Modification Regulating RNA Life. BioMed Research International. 11 May 2015; Vol. 2015, No. 968127; pages 1-13; page 3,	1, 3/1, 10-11, 13/10-11
-- Y	column 2, paragraph 3; DOI: 10.1155/2015/968127	2, 3/2, 12/10-11
Y –	(KOBS, G) Cloning Blunt-End DNA Fragments Into the pGEM®-T Vector Systems. Promega Notes Magazine. 1997; Vol. 62, No. 15; pages 1-5; page 3, paragraph 1	2, 3/2
Y	WO 2005/115481 A2 (ALNYLAM PHARMACEUTICALS INC.) 08 December 2005; page 2, lines 12-13; page 3, lines 12-16	12/10-11

☐ Further documents are listed in the continuation of Box C. ☐ 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
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority 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
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 September 2019 (16.09.2019)	10 OCT 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300