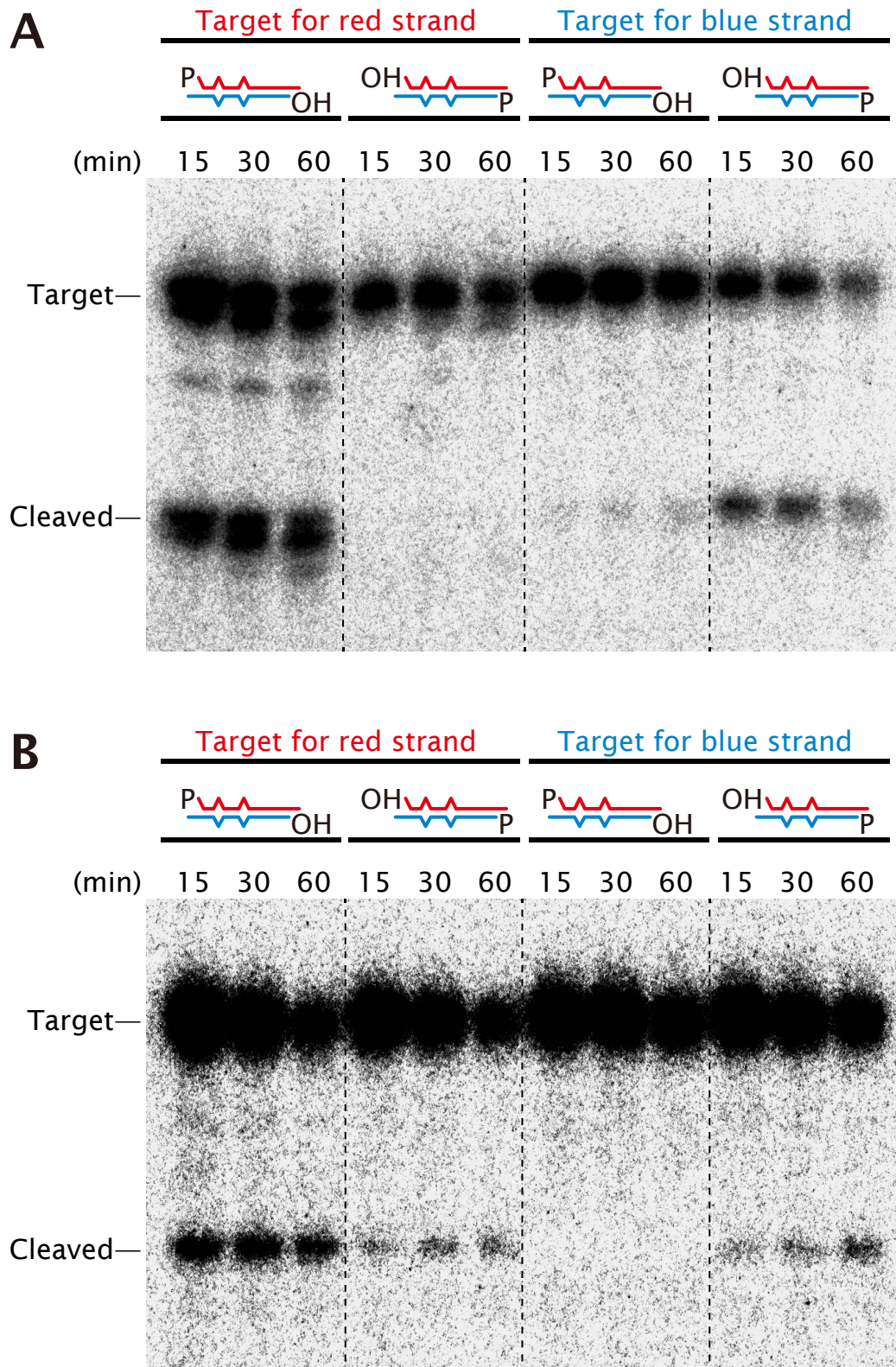


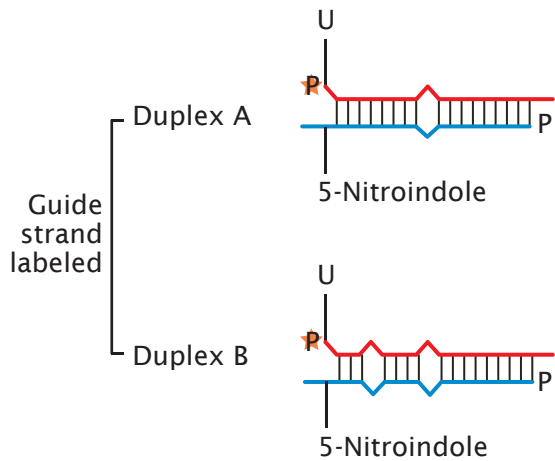
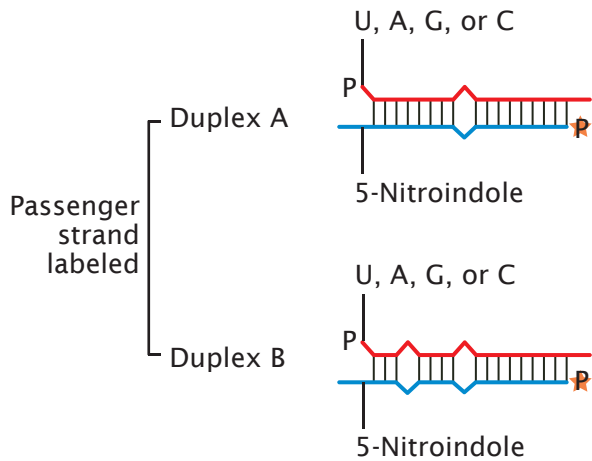
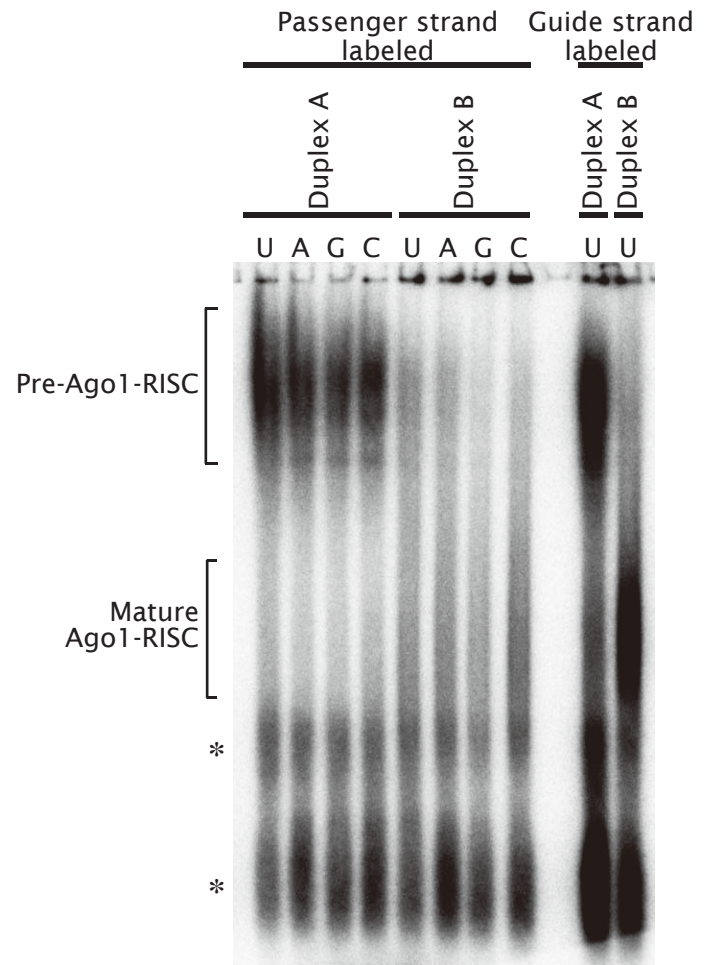
Supplementary Figure 1. Properties of 5'-dT small RNA duplexes

(A) Pre-phosphorylated 5'-dT duplexes are assembled into Ago1-RISC like pre-phosphorylated 5'-U small RNA duplexes. Duplex A and B with pre-phosphorylated 5'-U or 5'-dT and 3' radiolabel on the guide strand were incubated in lysate and pre- and mature Ago1-RISC were separated on agarose native gel. Asterisks indicate complexes previously shown to be unrelated to the Ago1-RISC assembly pathway (Kawamata et al., 2009; corresponding to complexes III and IV). Formation of these complexes was more apparent with 5'-P-dT duplexes than with 5'-P-U duplexes, but showed no correlation with the amounts of pre-Ago1-RISC and mature Ago1-RISC formed. At this point, the nature of these non-specific complexes remains unclear (perhaps related to RNA degradation), but does not affect our experiments and conclusions. (B) The 5'-dT duplex is barely phosphorylated in our standard experimental condition. Duplex A with 5'-OH-dT and 3' radiolabel on the guide strand was incubated in *dcr-2* embryo lysate. The 5'-dT duplex remained unphosphorylated at least for 60 min. Note that duplexes were incubated for 30 min in Figure 1. (C) 5'-dT duplexes behave similarly to phosphorylation-resistant 5'-MeO-dT duplexes. Duplex A and B with 5'-OH-U, 5'-OH-dT or 5'-MeO-dT and 3' radiolabel on the guide strand were incubated in *dcr-2* embryo lysate. The 5'-OH-U duplex B produced mature RISC, indicative of 5' phosphorylation, whereas no mature RISC was detected with 5'-OH-dT or 5'-MeO-dT duplex B. The sequences and structures of the duplexes are shown in Supplementary Table 3.



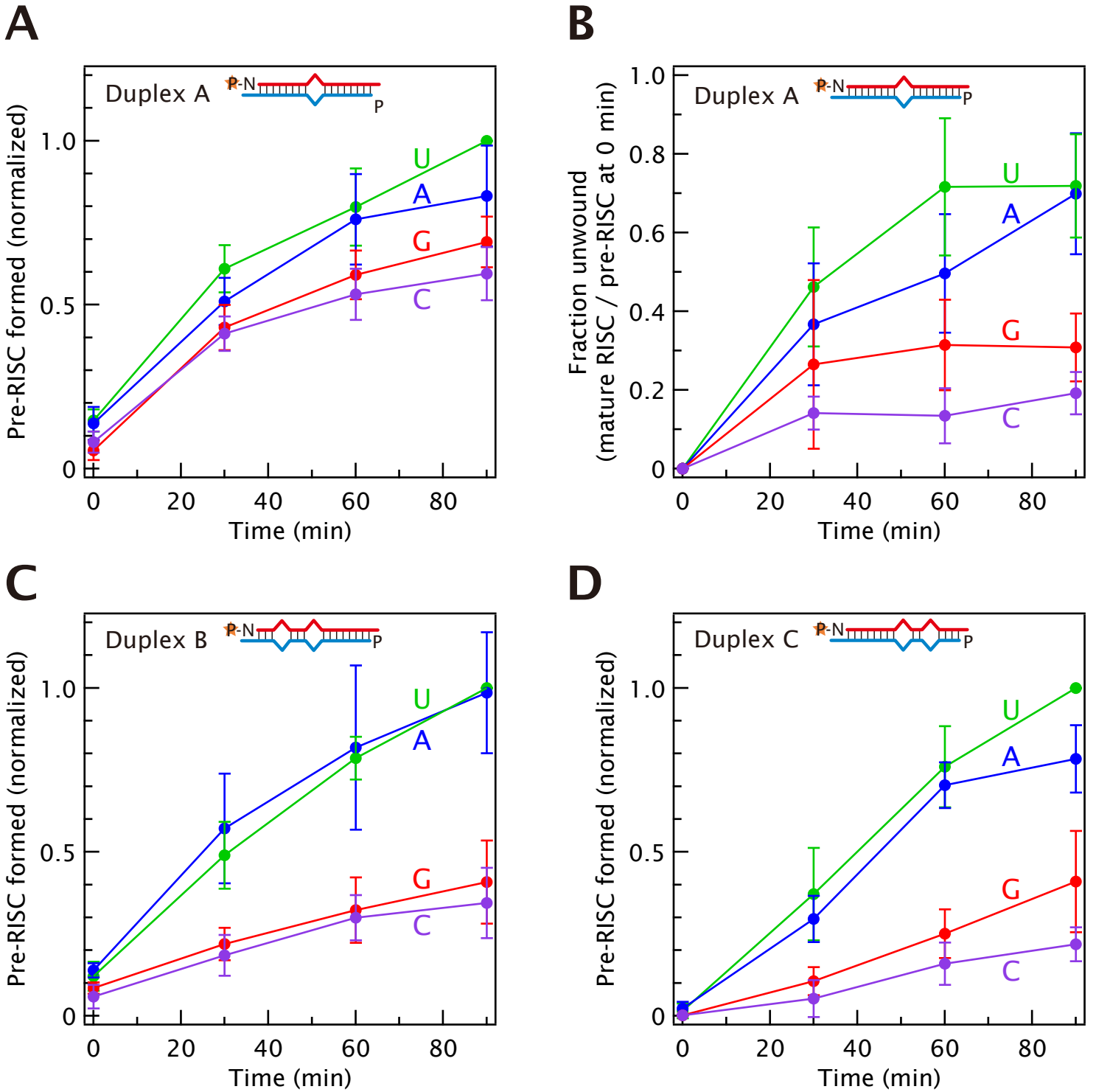
Supplementary Figure 2. 5' phosphate of the guide strand is strictly required for RISC assembly.

(A and B) Target cleavage assays for each of the two strands of (A) duplex B and (B) a luciferase-derived unrelated duplex. In both cases, the roles of the two strands were flipped when only the blue strands (originally the “passenger strands”) bore the 5' phosphates, agreeing well with the native gel analyses (Fig. 1). The sequences and structures of the duplexes are shown in Supplementary Table 3.

A**B**

Supplementary Figure 3. Introduction of 5-nitroindole on the passenger strand did not affect the functional asymmetry of the duplexes.

(A) Schematic representation of the duplexes used in B. Either the guide strand (red) or the passenger strand (blue) was 5' radiolabeled (denoted with stars). (B) Duplex A and Duplex B derivatives with 5' U, A, G or C on the guide strand and 5-nitroindole at position 19 of the passenger strand were incubated in lysate and pre- and mature Ago1-RISC were separated on agarose native gel. The sequences and structures of the duplexes are shown in Supplementary Table 3.



Supplementary Figure 4. Quantitative analysis of the gel-shift assays in Figure 2C-F.

(A) Quantification of Figure 2C from three independent trials. (B) Quantification of Figure 2D from four independent trials. (C) Quantification of Figure 2E from five independent trials. (D) Quantification of Figure 2F from three independent trials. The data in A, C and D are normalized to the amount of pre-Ago1-RISC formed with the 5' U duplex at 90 min. Mean values \pm standard deviations are shown.

Supplementary Table 1. Synthetic small RNA duplexes used in Figure 1.

The guide strand (red) of each small RNA duplex is derived from *Drosophila let-7* miRNA. ³²P-radiolabeled phosphates are in orange. Deoxythymidines (dT) are in purple.

Fig 1A - 1	Duplex A	5' -PO ₄ - TGAGGUAGUUGGUUGUAUAGpa -H-3' 3' -OH-UCUCUCCAUCAUCCAACAUA T -PO ₄ -5'
Fig 1A - 2	Duplex A	5' -PO ₄ - TGAGGUAGUUGGUUGUAUAGpa -H-3' 3' -OH-UCUCUCCAUCAUCCAACAUA T -OH-5'
Fig 1A - 3	Duplex B	5' -PO ₄ - TGAGGUAGUUGGUUGUAUAGpa -H-3' 3' -OH-UCUCUC GA UCAUCCAACAUA T -PO ₄ -5'
Fig 1A - 4	Duplex B	5' -PO ₄ - TGAGGUAGUUGGUUGUAUAGpa -H-3' 3' -OH-UCUCUC GA UCAUCCAACAUA T -OH-5'
Fig 1B - 1	Duplex A	5' -PO ₄ - TGAGGUAGUUGGUUGUAUAGA -OH-3' 3' - H- ap CUCUCCAUCAUCCAACAUA T -PO ₄ -5'
Fig 1B - 2	Duplex A	5' -PO ₄ - TGAGGUAGUUGGUUGUAUAGA -OH-3' 3' - H- ap CUCUCCAUCAUCCAACAUA T -OH-5'
Fig 1B - 3	Duplex B	5' -PO ₄ - TGAGGUAGUUGGUUGUAUAGA -OH-3' 3' - H- ap CUCUC GA UCAUCCAACAUA T -PO ₄ -5'
Fig 1B - 4	Duplex B	5' -PO ₄ - TGAGGUAGUUGGUUGUAUAGA -OH-3' 3' - H- ap CUCUC GA UCAUCCAACAUA T -OH-5'
Fig 1C - 1	Duplex A	5' -OH- TGAGGUAGUUGGUUGUAUAGpa -H-3' 3' -OH-UCUCUCCAUCAUCCAACAUA T -PO ₄ -5'
Fig 1C - 2	Duplex A	5' -OH- TGAGGUAGUUGGUUGUAUAGpa -H-3' 3' -OH-UCUCUCCAUCAUCCAACAUA T -OH-5'
Fig 1C - 3	Duplex B	5' -OH- TGAGGUAGUUGGUUGUAUAGpa -H-3' 3' -OH-UCUCUC GA UCAUCCAACAUA T -PO ₄ -5'
Fig 1C - 4	Duplex B	5' -OH- TGAGGUAGUUGGUUGUAUAGpa -H-3' 3' -OH-UCUCUC GA UCAUCCAACAUA T -OH-5'
Fig 1D - 1	Duplex A	5' -OH- TGAGGUAGUUGGUUGUAUAGA -OH-3' 3' - H- ap CUCUCCAUCAUCCAACAUA T -PO ₄ -5'
Fig 1D - 2	Duplex A	5' -OH- TGAGGUAGUUGGUUGUAUAGA -OH-3' 3' - H- ap CUCUCCAUCAUCCAACAUA T -OH-5'
Fig 1D - 3	Duplex B	5' -OH- TGAGGUAGUUGGUUGUAUAGA -OH-3' 3' - H- ap CUCUC GA UCAUCCAACAUA T -PO ₄ -5'
Fig 1D - 4	Duplex B	5' -OH- TGAGGUAGUUGGUUGUAUAGA -OH-3' 3' - H- ap CUCUC GA UCAUCCAACAUA T -OH-5'

Supplementary Table 2. Synthetic small RNA duplexes used in Figure 2.

The guide strand (red) of each small RNA duplex is derived from *Drosophila let-7* miRNA. N (green) denotes U, A, G, or C at the 5' end of the guide strand. "i" represents 5-nitroindole. ³²P-radiolabeled phosphates are in orange.

Fig. 2C and D	Duplex A	5' - ³² P ₄ -NGAGGUAGUUGGUUGUAUAGU-OH-3' 3' -OH-UCiCUCCAUCAUCCAACAUAU-PO ₄ -5'
Fig 2E	Duplex B	5' - ³² P ₄ -NGAGGUAGUUGGUUGUAUAGU-OH-3' 3' -OH-UCiCUCGAUCAUCCAACAUAU-PO ₄ -5'
Fig. 2F	Duplex C	5' - ³² P ₄ -NGAGGUAGUUGGUUGUAUAGU-OH-3' 3' -OH-UCiCUCCAUCAUCCUACAUAU-PO ₄ -5'

Supplementary Table 3. Synthetic small RNA duplexes used in Supplementary Fig. 1, Supplementary Fig. 2, and Supplementary Fig. 3.

The guide strand (red) of each small RNA duplex is derived from *Drosophila let-7* miRNA. ³²P-radiolabeled phosphates are in orange. Deoxythymidines (dT) are in purple. N (green) denotes U, A, G, or C. “i” represents 5-nitroindole.

Supplementary Fig. 1A	Duplex A	5' -PO ₄ -UGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCCAUCAUCCAACAUAU-PO ₄ -5'
Supplementary Fig. 1A	Duplex A	5' -PO ₄ -TGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCCAUCAUCCAACAUAU-PO ₄ -5'
Supplementary Fig. 1A	Duplex B	5' -PO ₄ -UGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCGAUCAUCCAACAUAU-PO ₄ -5'
Supplementary Fig. 1A	Duplex B	5' -PO ₄ -TGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCGAUCAUCCAACAUAU-PO ₄ -5'
Supplementary Fig. 1B	Duplex A	5' -OH-UGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCCAUCAUCCAACAUAU-PO ₄ -5'
Supplementary Fig. 1B	Duplex A	5' -OH-TGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCCAUCAUCCAACAUAU-PO ₄ -5'
Supplementary Fig. 1C	Duplex A	5' -OH-UGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCCAUCAUCCAACAUAU-PO ₄ -5'
Supplementary Fig. 1C	Duplex A	5' -OH-TGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCCAUCAUCCAACAUAU-PO ₄ -5'
Supplementary Fig. 1C	Duplex A	5' -MeO-TGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCCAUCAUCCAACAUAU-PO ₄ -5'
Supplementary Fig. 1C	Duplex B	5' -OH-UGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCGAUCAUCCAACAUAU-PO ₄ -5'
Supplementary Fig. 1C	Duplex B	5' -OH-TGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCGAUCAUCCAACAUAU-PO ₄ -5'
Supplementary Fig. 1C	Duplex B	5' -MeO-TGAGGUAGUUGGUUGUAUAGpA-H-3' 3' -OH-UCUCUCGAUCAUCCAACAUAU-PO ₄ -5'

Supplementary Fig. 2A	Duplex B (let-7)	5'-PO ₄ - TGAGGUAGUUGGUUGUAUAGU -OH-3' 3'-OH-UCUCUCCAUCAUCCAACAUA T -OH-5'
Supplementary Fig. 2A	Duplex B (let-7)	5'-OH- TGAGGUAGUUGGUUGUAUAGU -OH-3' 3'-OH-UCUCUC GAUCAUCCAACAUA T -PO ₄ -5'
Supplementary Fig. 2B	luciferase-derived	5'-PO ₄ - TCGAAGUAUUCGCGUACAUU -OH-3' 3'-OH-UUUGUAUCAUGGCGCGCAU T -OH-5'
Supplementary Fig. 2B	luciferase-derived	5'-OH- TCGAAGUAUUCGCGUACAUU -OH-3' 3'-OH-UUUGUAUCAUGGCGCGCAU T -PO ₄ -5'
Supplementary Fig. 3	Duplex A	5'-PO ₄ - NGAGGUAGUUGGUUGUAUAGU -OH-3' 3'-OH-UC i CUCCAUCAUCCAACAUAU- PO ₄ -5'
Supplementary Fig. 3	Duplex B	5'-PO ₄ - NGAGGUAGUUGGUUGUAUAGU -OH-3' 3'-OH-UC i CUCGAUCAUCCAACAUAU- PO ₄ -5'
Supplementary Fig. 3	Duplex A	5'- PO ₄ - UGAGGUAGUUGGUUGUAUAGU -OH-3' 3'-OH-UC i CUCCAUCAUCCAACAUAU- PO ₄ -5'
Supplementary Fig. 3	Duplex B	5'- PO ₄ - UGAGGUAGUUGGUUGUAUAGU -OH-3' 3'-OH-UC i CUCGAUCAUCCAACAUAU- PO ₄ -5'