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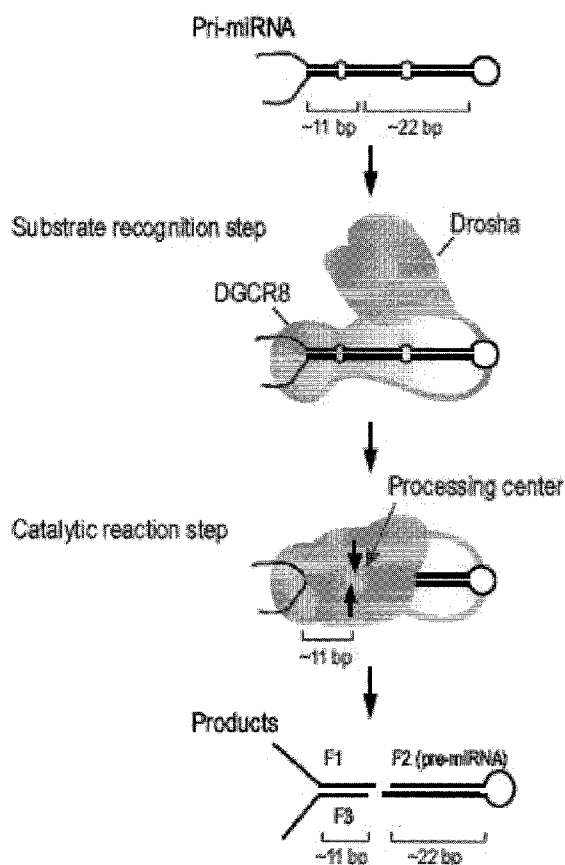
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(54) Title: THE RECOMBINANT PRIMARY-MICRORNA MOLECULE FOR RNA INTERFERENCE



(57) Abstract: The present invention relates to a preparing method for a recombinant primary-microRNA molecule for RNA interference, more precisely, a preparing method for an accurate and efficient primary-microRNA having corresponding sequence to its target gene. The present inventors confirmed that the cleavage site of pri-miRNA was not -22 bp from the terminal loop but -11 bp from the single strand (basal segments). Therefore, based on the new founding, the method of the invention can be effectively used for the generation of effective primary- microRNA for RNA interference.

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【DESCRIPTION】**【Invention Title】****THE RECOMBINANT PRIMARY-MICRORNA MOLECULE FOR RNA INTERFERENCE**

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【Technical Field】

The present invention relates to a preparing method for the recombinant primary-microRNA molecule for RNA interference, more precisely a preparing method for the efficient and accurate primary microRNA for interference having corresponding sequence with its target gene.

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【Background Art】

Small RNAs (referred as "sRNA" hereinafter) are ribonucleic acids of 17-25 nucleotides (referred as "nt" hereinafter) in length which regulate in vivo gene expression. According to their generation pathway, sRNAs are divided into microRNAs (referred as "miRNA" hereinafter) and small interfering RNAs (referred as "siRNA" hereinafter). MiRNAs are generated from partially double-stranded hairpin RNA while siRNAs are generated from long double stranded RNA (referred as "dsRNA" hereinafter). In general, it is miRNA who plays an important role in various regulatory mechanisms, whereas siRNA is a experimentally manipulated factor for regulation of gene

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expression. MiRNAs are endogenous molecules which inhibit protein generation from mRNAs by base-pairing with their target messenger RNAs (referred as "mRNA" hereinafter). In the meantime, siRNAs are exogenous molecules which decompose mRNAs by base-pairing with their mRNAs having corresponding sequence with them. RNA interference (referred as "RNAi" hereinafter) indicates, in a wide sense, the suppression of a gene by sRNAs by the intracellular insertion of synthesized siRNAs or by inducing the generation of siRNAs in vivo using virus or plasmid vector (see Fig. 1).

MicroRNAs (miRNA) are single stranded RNA (referred as "ssRNA" hereinafter) molecules of 19 - 25 nt in length that are generated from endogenous hairpin-shaped transcripts (Bartel, D.P., *Cell* 116:281-297, 2004; Kim, V.N., *Mol. Cells*. 19:1-15, 2005). MiRNAs act as post-transcriptional gene suppressors by base-pairing with their target mRNAs and inducing either translational repression or mRNA destabilization.

A strong link between miRNA and cancer has recently been demonstrated, opening up a new area of investigation in the field of cancer biology (Esquela-kerscher, A. and Slack, FJ., *Nat. Rev. Cancer* 6(4):259-269, 2006). The expression of miRNAs dramatically changes during development and cell differentiation. MiRNA profiling has

been shown to faithfully reflect both developmental lineages and disease states (Lu, J. *et al.*, *Nature* 435:834-838, 2005). In order to further dissect the regulatory networks in which miRNAs function, it will be crucial to first understand how these molecules are generated and controlled (Kim, V.N. *Nat. Rev. Mol. Cell. Biol.* 6:376-385, 2005).

MiRNA biogenesis is initiated via transcription by RNA polymerase II (Cai. X., *et al.*, *RNA* 10:1957-1966, 2004; Kim, V.N. *Nat. Rev. Mol. Cell. Biol.* 6:376-385, 2005; Lee, Y., *et al.*, *EMBO J* 21:4663-4670, 2002; Lee, Y., *et al.*, *EMBO J* 23:4051-4060, 2004). The primary transcript (primary microRNA: referred as "pri-miRNA" hereinafter) is usually species of over several kilobases long and contain both a 5' cap and a poly A tail. Pri-miRNA processing is a critical step in miRNA biogenesis because it defines the miRNA sequences embedded in long pri-miRNAs by generating one end of the molecule. Pri-miRNAs are first cropped to release -65 nt of hairpin-shaped precursor (pre-miRNA) by a member of the ribonuclease III family, Drosha (Lee, Y., *et al.*, *Nature* 425:415-419, 2003) and microprocessor which is a complex formed by its co-factor DGCR8 (Gregory, R.I. *et al.*, *Nature* 432:235-240, 2004; Han, J., *et al.*, *Genes Dev.* 18:3016-3027, 2004; Landthaler, M., *et al.*, *Curr. Biol.* 14:2162-2167, 2004). Following this initial processing,

the resulting pre-miRNAs are exported by the nuclear transport factor, Exp5 (exportin-5), in the cytoplasm where Dicer, a cytoplasmic RNase III type protein, dices the transported pre-miRNAs to generate -22 nt miRNA duplexes (Bohnsack, M.T., et al., *RNA* 10:185-191, 2004; Lund, E., et al., *Science* 303:95-98, 2004; Yi, R., et al., *Genes Dev.* 17:3011-3016, 2003). One strand of the Dicer product remains as a mature miRNA and is then assembled into effector complex called miRNP (microribonucleoprotein) or miRISC (miRNA-induced silencing complex) (Khvorova, A., et al., *Cell* 115:209-216, 2003; Schwarz, D.S., et al., *Cell* 115:199-208, 2003).

RNA interference (RNAi), the gene silencing mechanism that is mediated by small RNAs, is now a powerful genetic tool in mammalian systems. Effective and stable gene knockdown can be achieved by the expression of short hairpin RNAs (referred as "shRNA" hereinafter) which are processed into small interfering RNAs (siRNAs). Recent breakthroughs in RNAi technology have been made by generating shRNA expression cassettes that can mimic a natural miRNA gene (Dickins, R.A., et al., *Nat. Genet.* 37:1289-1295, 2005; Silva, J.M. et al., *Nat. Genet.* 37:1281-1288, 2005; Zeng, Y., et al., *Mol. Cell* 9:1327-1333, 2002). MiRNA based shRNAs, driven by RNA polymerase II promoters, can induce efficient, stable and regulated

silencing in cultured cells as well as in animal models. The expression of such shRNAs is dependent upon the presence of miRNA biogenesis factors. Therefore, a mechanistic understanding of miRNA processing is crucial for the rational design of accurate and efficient shRNAs.

RNase III type proteins have major functions in RNA silencing pathways. RNase III cleaves double stranded RNA (dsRNA) in a staggered manner and creates a 2 nt overhang on the 3' end of its products (Kim, V.N. *Nat. Rev. Mol. Cell Biol.* 6:376-385, 2005; Tomari, Y., and Zamore, P.D., *Curr. Biol.* 15:R61-64, 2005). This enzyme family can be grouped into three classes based on their domain organization. Class 1 proteins are found in yeast and bacteria and have an RNase III domain (R^{III}D) and a dsRBD (double stranded RNA-binding domain). Drosha homologs belong to the class 2 grouping and possess tandem R^{III}IDs and a dsRBD, in addition to an extended N-terminus that contains a proline rich region and a serine/arginine rich region of unknown function. Dicer homologs, which are class 3 proteins within this enzyme family, contain two R^{III}IDs, one dsRBD and a long N-terminus. The N-terminal region of Dicer is composed of an RNA helicase/ATPase domain, a DUF283 domain and a PAZ domain.

RNase III proteins show a high degree of conservation in their catalytic domains and also share a basic action

mechanism. Two RIII₁Ds interact with each other to constitute a single processing center where two catalytic sites are placed closely and each of the two catalytic sites cleaves on one strand of an RNA duplex (Blaszczyk, M.T., et al., *Structure(Camb)* 9:1225-1236, 2001; Zhang, H., et al., *Cell* 118:57-68, 2004). Both the Dicer and Drosha enzymes form an intramolecular dimer of two RIII₁Ds (Han, J., et al., *Genes. Dev.* 18:3016-3027, 2004; Zhang, H., et al., *Cell* 118:57-68, 2004). The C-terminal RIII₁D (RIII₁Db), proximal to the dsRBD, cleaves the 5' strand of the hairpin, whereas the other RIII₁D (RIII₁Da) cleaves the 3' strand.

Despite the similarities in their basic modes of action, RNase III proteins are different in many interesting ways, particularly in their substrate specificities. Dicer will act on any dsRNA with a simple preference towards the terminus of the molecule and produce -22 nt fragments progressively from the terminus. The PAZ domain of Dicer interacts with the 3' overhang at the terminus and determines the processing site in a ruler-like fashion that measures -22 nt segments away from the terminus (Lingel, A., et al., *Nat. Struct. Mol. Biol.* 11:576-577, 2004; Ma, J.B., et al., *Nature* 429:318-322, 2004; Macrae, I.J., et al., *Science* 311:195-198, 2006; Song, J.J., et al., *Nat. Struct. Biol.* 10:1026-1032, 2003).

FIG. 1. Schematic diagram of pri-miRNA structure.

Although Drosha is the only known enzyme that can process a variety of pri-miRNAs, no common sequence motif has been found among the human pri-miRNA species. Thus, it is plausible that the Drosha-DGCR8 complex recognizes common structural features in these molecules. A typical animal pri-miRNA comprises a stem, a terminal loop and long flanking sequences. Zeng et al. have shown previously that a large terminal loop is in fact critical for processing and the cleavage site may be determined largely by the distance from this structure (2 helical turn) (Zeng, Y., et al., *EMBO J* 24:138-148, 2005). The sequences flanking the stem loop have also been shown to be important for efficient processing *in vitro* (Lee, Y., et al., *Nature* 425:415-419, 2003; Zeng, Y., et al., *EMBO J* 24:138-148, 2005) like *in vivo* (Chen, C.Z., et al., *Science* 303:83-86, 2004). It remains to be determined which protein is responsible for specific recognition of pri-miRNAs.

【Disclosure】

【Technical Problem】

It is an object of the present invention to provide a pri-microRNA responsible for effective mRNA silencing.

【Technical Solution】

To achieve the above object, the present invention provides a recombinant primary-microRNA (referred as "pri-miRNA" hereinafter) molecule containing serially a single strand tail; a lower stem of 8 - 14 bp; an upper stem harboring a target gene specific sequence; and a terminal loop.

The present invention also provides a recombinant pri-miRNA molecule containing serially a single strand tail; a lower stem of 8 - 14 bp; an upper stem harboring a target gene specific sequence; and a single strand head.

The present invention further provides a polynucleotide molecule encoding the nucleotide sequence of the recombinant pri-miRNA.

The present invention also provides a recombinant expression vector containing the polynucleotide molecule above.

The present invention also provides a therapeutic agent for disease containing the recombinant pri-miRNA molecule and/or the expression vector as an effective ingredient.

The present invention also provides a preparing method for the recombinant pri-miRNA molecule.

The present invention also provides a preparing method for the recombinant expression vector.

The terms of the invention are defined as follows;

Small RNA (referred as 'sRNA' hereinafter) is a RNA of 15 - 17 nt in length that regulates gene expression *in vivo*.

5 MicroRNA (referred as 'miRNA' hereinafter) is a single stranded RNA molecule of 19 - 25 nt in length which is generated from partially double stranded hairpin shaped RNA. MicroRNA plays an important role in variety of regulation systems including suppression of the translation
10 from mRNA by base-pairing specifically with its messenger RNA (referred as 'mRNA' hereinafter).

 Small interfering RNA (referred as 'siRNA' hereinafter) is generated from a long double stranded RNA and plays an important role in variety of regulation
15 systems including suppression of the translation from mRNA by base-pairing specifically with its messenger RNA, as miRNA does.

 Primary microRNA (referred as 'pri-miRNA' hereinafter) is generated from the transcription of DNA by
20 RNA polymerase II which has a hairpin-shaped structure of over several kb long and contains a 5' cap and a poly(A) tail.

 Precursor microRNA (referred as 'pre-miRNA' hereinafter) is a molecule having a hairpin-shaped

structure of 65 nt in length generated by cropping of pri-miRNA with Drosha-DGCR8 complex.

5' donor indicates the pri-miRNA harboring its mature sequence at the 5' strand of the hairpin.

5 3' donor indicates the pri-miRNA harboring its mature sequence at the 3' strand of the hairpin.

10 A single strand tail is the opposite area of a terminal loop of pri-miRNA and composed of two single strands whose 5' strands do not have corresponding sequences with their 3' strands.

A single strand head is a substitution for a single strand of pri-miRNA terminal loop which does not interact.

15 Hereinafter, the present invention is described in detail.

20 The present inventors collected pri-miRNAs of 110 nt in length from human and drosophila pri-miRNAs, then selected 280 human pri-miRNAs and 66 drosophila pri-miRNAs, excluding those that are predicted to have multiloops (see Table 1). And the secondary structures of those selected pri-miRNAs were predicted, and each nt was assigned with position numbers. To elucidate the general structures of those pri-miRNAs, thermodynamic features of pri-miRNAs were analyzed with measuring free energy of each part (see Fig. 2 - Fig. 5), followed by averaging the free energy values

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(see Fig. 2 and Fig. 3). As a result, the pri-miRNA was confirmed to serially consist of a single strand tail, an 11 bp long lower stem, an upper stem harboring its target gene specific sequence and a terminal loop. The upper stem contains two helical turns, while the lower stem includes one helical turn. The entire lengths of the upper and lower stems are approximately 33 bp and the terminal loop is about 16 nt long. Pri-miRNA molecules are cropped by Drosha-DGCR8 by -22 bp long from the terminal loop.

Based on the general structures of pri-miRNAs, as shown in Table 2, the present inventors induced mutation and pri-miRNA processing *in vitro*. To understand the cleavage by Drosha-DGCR8, mutants with elimination of the terminal loop were prepared (16-TL1, TL2). The mutant lacking a terminal loop was processed at the original site (see Fig. 7). Another mutant (16-TL3) with the substitution with a large internal loop was also processed at the original site by Drosha-DGCR8 complex (see Fig. 8). From the results were confirmed that the terminal loop structure itself is not necessary for pri-miRNA processing. The Drosha-DGCR8 complex may therefore process not only hairpin RNAs but also other substrates such as long dsRNAs with large internal loops.

The present inventors next generated an inverted hairpin mutant (16-TL4), in which a single strand tail and

a terminal loop were substituted, followed by Drosha-DGCR8 complex cleavage. As a result, this inverted hairpin variant was also processed at the original cleavage site (see Fig. 9). The sequences in the single strand tail

5 cleaved in the inverted hairpin structure were modified, resulting in mutants 16-TL5 and 16-TL6. From the Drosha-DGCR8 cleavage was confirmed that the variants were also processed at the original cleavage site (see Fig. 9).

10 Additionally, more inverted hairpin structured mutants were prepared based on miR-30 and miR-23 sequences (30-TL1, 23-TL1, and 23-TL2). From the Drosha-DGCR8 cleavage was confirmed that the variants were also processed at the original cleavage site (see Fig. 10 and Fig. 11). From the

15 above results was confirmed that the distance from the terminal loop is not imperative for cleavage site selection.

Then, a single strand tail was converted to produce another mutant. Particularly, a mutant with complete elimination of a single strand tail (16- Δ BS), a mutant with elimination of 5' strand (16-5'BS) and a mutant with
20 elimination of 3' strand (16-3'BS) were prepared, followed by Drosha-DGCR8 complex cleavage reaction. As a result, when the single strand tail was completely eliminated from pri-miRNA variant 16- Δ BS, processing was abolished. However, the variants 16-5'BS and 16-3'BS were processed at
25 the original sites (see Fig. 12). The above results

ABSTRACT OF THE DISCLOSURE

indicate that the single strand tail is critical for Dosha-DGCR8 complex processing. The variant (16-BS1) in which the single strand tail sequence was modified was processed at the original site by Dosha-DGCR8. In the meantime, the variant in which the single strand was converted into double strand was processed with less efficiency, compared with 16-BS1 (see Fig. 13), also supporting the above confirmation that it is the single-stranded nature of the basal segments, rather than nt sequence, that is critical for Dosha-DGCR8 complex processing.

More mutants were prepared by moving the loop-stem junctions for further processing assay. The present inventors introduced a deletion to the lower stem to reduce the distance by 4 bp from the single strand (16-L-4). As a result, the cleavage site by Dosha-DGCR8 complex was shifted by 4 bp away from the original site to the upper stem (see Fig. 14). Three upper stem mutants, 16-U+2, 16-U-2 and 16-U-6, were also prepared. Unlike the result of 16-L-4, they were cleaved at the original sites (see Fig. 15). Then, the present inventors introduced further deletions in the upper stem to generate variants with a small terminal loop (16-TL7) and a shorter stem (16-TL7/U-10, 16-ATL7/U-20). Their cleavage sites were 11 bp away from the single strand tail (see Fig. 16). The above results indicate that the distance from the terminal loop

is unlikely to be the major determinant of cleavage site selection. In the meantime, the cleavage site was determined by the distance exactly 11 bp away from the single strand tail toward the lower stem.

5 Drosha DGCR8 protein was linked to FLAG epitope, which was introduced into HEK293T cells. Silver staining and UV crosslinking were carried out. As a result, the binding activity of Drosha to RNA was unstable and weak (see Fig. 17). Drosha may interact with pri-miRNA
10 transiently whereas DGCR8 associates directly with pri-miRNA in a more stable manner. To further examine the mode of interaction between DGCR8 and RNA, UV-crosslinking experiments were carried out using FLAG-DGCR8 protein and various RNA molecules (pri-miR-30a, siRNA duplex, ssRNA (23
15 bp), dsRNA (80 bp), and pre-miR-30a). As a result, unlabeled pri-miR-30a competed with labeled pri-miR-30a, but other RNA molecules did not compete efficiently in this reaction (see Fig. 19). Pre-miR-30a was found to have barely competed with pri-miR-30a under these conditions,
20 indicating that the main binding site for DGCR8 resides outside the upper stem and the terminal loop. Long dsRNA has a relatively high affinity to DGCR8 and 23 nt ssRNA is also capable of competing with pri-miR-30a weakly but not efficiently (see Fig. 19), suggesting that DGCR8 may
25 interact with pri-miRNAs by recognizing both ssRNA and

dsRNA structures. The present inventors also carried out crosslinking experiments using mutated pri-miRNAs (m16- Δ BS, m16-TL7, m16-TL7/U-10, and m16-TL7/U-20) as competitors. As a result, the basal segment mutant m16- Δ BS could not compete with wild type RNA for binding to DGCR8. The pri-miRNA variant with a small terminal loop (16-TL7) was either impaired with binding or not able to compete with wild type RNA (see Fig. 20). In addition, short-stem mutants (m16-TL7/U-10, m16-TL7/U-20) were also tested. As the stem becomes shorter, the mutants competed less efficiently than the longer mutants (see Fig. 20).

The present inventors generated an artificial pri-miRNA molecule bearing no sequence homology to any known pri-miRNAs, which has the structure of "ssRNA tail-3-helical turns-ssRNA tail" (see Fig. 21). The artificial substrate was cleaved either at -11 bp from the left junction or at -11 bp from the right junction (see Fig. 21). Then, the molecular structure of the artificial substrate was modified (see Fig. 22). The simple dsRNA was not cleaved by Drosha-DGCR8 complex and the artificial pri-miRNA molecule bearing a single strand alone was cleaved at 11 bp away from the junction by Drosha-DGCR8 complex (see Fig. 22). To examine whether DGCR8 binds to the ssRNA tails, the relative affinity of these RNAs for DGCR8 protein was determined. As a result, the duplexes

containing ssRNA tails displayed high affinity to DGCR8, whereas a simple duplex could not compete for this interaction efficiently (see Fig. 23).

From this invention was provided a novel pri-miRNA processing model. It has been reported that the Drosha-DGCR8 complex cleavage site might be at 22 bp away from the terminal loop of pri-miRNA. However, the results of the experiments of the invention confirmed that the cleavage site is at 11 bp from the single strand segment and the single strand is critical factor for pri-miRNA processing (see Fig. 24).

The present inventors further prepared shRNAs responsible for RNA silencing by targeting luciferase (see Fig. 27 and Figs 29 - 32) and confirmed that the shRNA could effectively reduce luciferase activity (see Fig. 28 and Fig. 33).

The present invention provides a recombinant pri-miRNA molecule harboring a single strand tail, a 8 - 14 bp long lower stem, an upper stem containing a target gene specific sequence and a terminal loop in that order.

The length of the single strand tail of the recombinant pri-miRNA is preferably more than 5 nt and more preferably 8 - 15 nt. The single strand tail of the recombinant pri-miRNA molecule preferably contains one of

or both of 5' end strand and/or 3' end strand, but not always limited thereto. It is also preferred for the 5' end strand and 3' end strand of the single strand tail does not correspond with each other.

5 The length of the lower stem of the recombinant pri-miRNA is preferably 8 - 14 bp, and more preferably 11 bp. The lower stem preferably contains a mis-match consisting of 1 or 2 internal loop(s) and/or bulge(s), and preferably contains one mis-match but not always limited thereto.

10 The upper stem of the recombinant pri-miRNA molecule of the invention encodes target mRNA sequences of 5' strand or 3' strand. Target mRNA sequences can be any mRNA sequence that is able to be used for RNA silencing. The length of the upper stem is preferably 8 - 30 bp and more
15 preferably 17 - 24 bp, but not always limited thereto. The upper stem preferably contains a mis-match consisting of one to three internal loop(s) and/or bulge(s) and preferably contains one to two mis-matches, but not always limited thereto. It is also preferred to use RNA bearing a
20 mis-match (G-U pair, internal loop, or bulge) in the site having a target gene specific sequence for RNA interference. The mis-match preferably resides at 9 - 15 nt, and the preferable numbers of the mismatch is 1 - 4 and the preferable size of the mismatch is 1 nt - 3 nt. These

conditions are commonly applied to every RNA available for RNA interference using pri-miRNA.

The terminal loop of the recombinant pri-miRNA is characterized by the binding of double strand of the upper stem. The length of the terminal loop is preferably 1 - 20 nt and more preferably 4 - 18 nt but not always limited thereto.

The junction of the upper stem and the lower stem of the recombinant pri-miRNA is thermodynamically unstable, compared with other parts, and cleaved by Drosha-DGCR8 complex.

The present invention also provides a recombinant pri-miRNA molecule containing serially a single tail, a 8 - 14 bp long lower stem, a upper stem harboring a target gene specific sequence and a single strand head.

The length of the single strand tail and the single strand head of the above recombinant pri-miRNA molecule is preferably more than 5 nt and more preferably 8 - 15 nt and most preferably 8 - 14 nt. The single strand tail and the single strand head of the recombinant pri-miRNA preferably contain one of or both of 5' end strand and/or 3' end strand, but not always limited thereto. It is preferred that the 5' end strand of the single strand tail does not correspond to the 3' end strand.

The length of the lower stem of the above recombinant pri-miRNA molecule is preferably 8 - 14 bp and more preferably 11 bp. The lower stem preferably contains a mismatch consisting of one or two internal loop(s) and/or bulge(s) and the preferable number of the mismatch is one but not always limited thereto.

The upper stem of the recombinant pri-miRNA molecule encodes its target mRNA sequence in its 5' strand or 3' strand. The target mRNA sequence can be any mRNA sequence available for RNA silencing. The length of the upper stem is preferably 8 - 30 bp and more preferably 10 - 22 bp but not always limited thereto. The upper stem preferably contains a mismatch consisting of one - three internal loop(s) and/or bulge(s) and the preferable number of the mismatch is 1 - 2 but not always limited thereto. It also includes RNA bearing a mismatch (G-U pair, internal loop, or bulge) in its target gene specific sequence region for RNA interference. The mismatch preferably resides at 9 - 15 nt and the number of mismatch is preferably 1 to 4 and the size of it is 1 nt - 3 nt. These conditions are commonly applied to every RNA used for RNA interference using pri-miRNA.

The junction of the upper stem and the lower stem of the recombinant pri-miRNA is thermodynamically unstable and cleaved by Drosha-DGCR8 complex.

The present invention further provides a polynucleotide molecule encoding the nucleotide sequence of the recombinant pri-miRNA molecule.

5 The gene was preferably designed to encode successively the sequence of 5' end; the sense sequence of a lower stem; the sense sequence of an upper stem harboring its target gene specific sequence; the sequence of a terminal loop; an antisense sequence of an upper stem
10 containing a target gene specific sequence; the antisense sequence of a lower stem; and the sequence of 3' end.

The polynucleotide molecule was preferably designed for annealing of a lower strand encoding successively the sequence of 5' end; the sense sequence of a lower stem; the
15 sense sequence of an upper stem containing a target gene specific sequence; an upper strand encoding serially 3' end sequence; the antisense sequence of a lower stem; the antisense sequence of an upper stem containing a target gene specific sequence; and the sequence of 5' end.

20 The polynucleotide molecule encoding the recombinant pri-miRNA molecule can be synthesized by the standard method well-informed to those in the art using an automatic DNA synthesizer (Bioserch or Applied Biosystems). The synthesized pri-miRNA precursor can be used as it is or
25 cloned into an expression vector by the conventional method

well-known to those in the art. The recombinant pri-miRNA molecule of the invention is supposed to be intracellular transported *in vivo* or *in vitro* for inducing RNA sequencing of its target mRNA.

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The present invention also provides a recombinant expression vector containing the above polynucleotide molecule.

10 The recombinant expression vector of the invention can be designed by the conventional recombinant DNA preparing method known to those in the art. The recombinant expression vector can be selected from a group consisting of plasmid, lentivirus vector, retrovirus vector and adenovirus vector, which can be used for the
15 duplication and expression of mammalian cells and other target cells. A gene encoding the nucleotide sequence of the recombinant pri-miRNA can be synthesized to be inserted into an expression vector.

20 Herein, RNA polymerase II promoter was selected as a recombinant expression vector. For the transcription of a short transcript, RNA polymerase III promoter has been used and U6 promoter and/or H1 promoter is the representative promoter. However, it is not easy to control the RNA polymerase III promoter. The RNA polymerase II promoter of
25 the present invention ought to be easily regulated and to

induce tissue-specific expression with the insertion of a secretion sequence in front of the promoter. The RNA polymerase II promoter of the invention is preferably selected from a group consisting of cytomegalovirus immediate early promoter, SV40 promoter, human immunodeficiency virus LTR and c-myc promoter.

The present invention also provides a therapeutic agent containing the recombinant pri-miRNA molecule and/or the recombinant expression vector.

The recombinant pri-miRNA molecule and/or the recombinant expression vector of the invention can be included in a pharmaceutical composition for the treatment of disease. The therapeutic agent of the present invention can include pri-miRNA alone and together with a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier is exemplified by solvent appropriate for pharmaceutical administration, dispersive media, coating, antibacterial agent and antimicrobial agent, isotonic agent and absorption retarder. Besides, an adjuvant compound can also be additionally included.

The therapeutic agent of the present invention can be formulated to be appropriate for the administration pathway. The administration pathway is exemplified by oral administration or parenteral administration such as

intravenous injection, intrablood injection, hypodermic injection, inhalation, percutaneous (local) administration and intramucous injection and intrarectal injection. A solution or suspension for the parenteral administration such as intrablood or hypodermic injection includes an aseptic diluent such as solvent for injection, saline, fixative oil, polyethylene glycol, glycerin, propylene glycol or other synthetic solvents; an antibacterial agent such as benzine alcohol or methyl paraben; an anti-oxidant agent such as ascorbic acid or sodium sulfite; a chelating agent such as ethylenediaminetetraacetic acid; a buffer such as acetate, citrate or phosphate; and an isotonic regulator such as sodium chloride or dextrose. PH can be regulated by acid or base such as HCl or NaOH. Formulations for parenteral administration can be prepared in multiple dose vials made of ampoule, disposable syringe, glass or plastic.

Aseptic powder can be separately prepared to be added instantly to the suspension for injection such as aseptic aqueous solution or dispersion and aseptic injection or dispersion. The acceptable carrier for the intravenous injection can include saline, bacteriostatic water, CremophorELTM (BASF, Parsippany, NJ) or PBS. In every cases, all the therapeutic agents have to be sterilized and in the form of proper solution for injection. They have to be

stable during the preparation and storage and well-preserved under the activity of microorganism including bacteria and fungi. A carrier can be one of water, ethanol, polyol (glycerol, propylene glycol, liquid polyethylene glycol, etc.) or a mixing solvent thereof or a dispersion medium. For example, a coating agent like lecithin can keep the granularity of dispersion and a surfactant can keep a proper fluidity. The activity of a microorganism can be prevented by various antibacterial and antifungal agents such as paraben, chlorobutanol, phenol, ascorbic acid, thimerosal, etc. The therapeutic agent of the invention can additionally include polyvalent alcohol such as saccharose, manitol and sorbitol and isotonic agent like sodium chloride. To prolong the absorption of the injectable composition, an absorption retarder such as aluminum monostearate and gelatin can be included in the therapeutic agent.

An aseptic injection can be prepared by adding one or more active compounds mentioned above to a proper solvent and sterilizing thereof. Generally, a dispersion can be prepared by mixing an active compound, a basic dispersion medium and a sterilized vehicle containing other necessary compounds. Aseptic powders for the injection can be prepared by vacuum drying and freeze-drying, by which

precisely an active ingredient and target compounds can be obtained from the pre-sterilized filtered solution.

A therapeutic agent for oral administration includes an inactive diluent or an edible carrier. For oral administration, an active ingredient is added to an excipient, resulting in tablets, troches and gelatin capsules. The therapeutic agent for oral administration can also be prepared using a liquid carrier to be used as a mouth washer. Pharmaceutically acceptable binders and/or adjuvants can be additionally included. Tablets, pills, capsules and troches can contain one of or a similarly-functioning mixture of the following components; for example, a binder such as cellulose, gum tracaganth or gelatin; an excipient such as starch or lactose; a disintegrating agent such as alginic acid, PrimogelTM or corn starch; a lubricant such as magnesium stearate or SterotesTM; a gildant such as silicon dioxide; a sweetener such as sucrose or saccharin; a flavor such as peppermint, methyl salisylate or orange flavor. It is preferred to prepare such therapeutic agents for oral or parenteral administration in the dosage-unit form for easy administration and uniformity.

The toxicity and therapeutic effect of the therapeutic agent of the invention were calculated by measuring LD₅₀ (the dosage that is able to kill 50% of

experimental group) and ED_{50} (the dosage that is able to induce treatment effect on 50% of experimental group), which were carried out based on the standard pharmaceutical procedure by using cell culture or test animals. The ratio of dosage having cytotoxicity to the dosage having treatment effect becomes treatment index, which is the ratio of LD_{50} to ED_{50} . A therapeutic agent having high treatment index is preferred. A therapeutic agent showing toxic side effects might be used. Thus, to reduce the possibility of using such toxic agent, a delivery system has to be designed to reduce the damage area of non-infected cells by targeting.

The effective dosage of the therapeutic agent of the invention is determined by the expression and activity of the target molecule (pri-miRNA molecule). When the pri-miRNA molecule is administered to an animal, lower dosage is first prescribed by a doctor, a vet or a researcher and then the dosage can be increased until proper reaction is induced. And a dosage can be varied with the activity of a specific compound, age, weight, health condition, gender and dietary habit, administration frequency and pathway, excretion, ingredients and other factors affecting the expression and activity of the composition.

The therapeutic agent of the present invention can be applied to cancer, viral infection, genetic disease,

metabolic disease, immune disease, neurodegenerative disease and ophthalmology disease. It is possible for those in the art to select a protein which is closely involved in the cause or the metabolism of a disease as a target protein and analyzed the gene sequence encoding the target protein, so that they can design pri-miRNA of the invention and used for effective gene silencing of the target protein (Dykxhoorn, D.M., et al., *Gene Ther.* 13:541-552, 2006; Gartel, A.L., et al., *Biomol. Eng.* 23:17-34, 2006; Paulson, H. *Neurology* 66:S114-117, 2006; Rossi, J.J. *Biotechniques Suppl*, 25-29, 2006).

The present invention also provides a preparing method for the pri-microRNA molecule.

The present invention provides a preparing method for the recombinant pri-microRNA molecule comprising the following steps:

- 1) Selecting mRNAs of target genes;
- 2) Determining the pri-microRNA to be composed of 5' end sequence; sense sequence of a lower stem; sense sequence of a upper stem containing a target gene specific sequence; a terminal loop sequence; antisense sequence of a upper stem containing a target gene sequence; antisense sequence of a lower stem; and 3' end sequence successively;

3) Synthesizing the pri-miRNA molecule of step 2);
and

4) Annealing the pri-miRNA synthesized in step 3) to
predict the secondary structure.

5

The present invention also provides a preparing
method for the recombinant expression vector.

The present invention provides a preparing method for
the recombinant expression vector comprising the following
steps:

10

1) Selecting mRNAs of target genes;

2) Determining the pri-miRNA to be composed of 5' end
sequence; sense sequence of a lower stem; sense sequence of
a upper stem containing a target gene specific sequence; a
terminal loop sequence; antisense sequence of a upper stem
containing a target gene sequence; antisense sequence of a
lower stem; and 3' end sequence successively;

15

3) Synthesizing a single stranded DNA strand
corresponding to the recombinant pri-mRNA of step 2) and an
antisense single stranded DNA strand thereof;

20

4) Annealing the single stranded DNA strands
synthesized in step 3) to prepare a double stranded DNA;
and

5) Linking the double stranded DNA of step 4)
operably to the promoter of an expression vector.

25

The present invention further provides a preparing method for the recombinant expression vector comprising the following steps:

1) Selecting mRNAs of target genes;

5 2) Determining the sequences of the upper strand RNA molecule harboring successively 5' end sequence; sense sequence of a lower stem; sense sequence of a upper stem containing a target gene specific sequence; and 3' end sequence of a upper stem and the lower strand RNA molecule
10 harboring serially 3' end sequence, antisense sequence of a lower stem, antisense sequence of a upper stem harboring a target gene specific sequence and 5' end sequence of a lower stem;

15 3) Synthesizing a single stranded DNA and its antisense single stranded DNA corresponding to the nucleotide sequences of the upper strand RNA and the lower strand RNA determined in step 2);

20 4) Annealing the single stranded DNA strands synthesized in step 3) to form a double stranded DNA molecule by base-pairing; and

5) Linking the double stranded DNA of step 4) operably to the promoter of an expression vector.

According to the above preparing method, the expression vector of the step 5) is preferably selected

from a group consisting of plasmid, lentivirus vector, retrovirus vector and adenovirus vector.

【Description of Drawings】

5 The application of the preferred embodiments of the present invention is best understood with reference to the accompanying drawings, wherein:

10 Fig. 1 is a concept map illustrating RNA interference of siRNA and microRNA (referred as "miRNA" hereinafter),

 Fig. 2 is a set of graphs and diagrams illustrating thermodynamic stability of miRNA encoding mRNA sequence of human 5' donor:

15 A: thermodynamic stability profiles;

 Upper panel: graph of the thermodynamic stability profiling at each position;

 Center panel: graph of the standard deviation at each position;

20 Lower panel: graph of the number of mismatches such as an internal loop or a bulge at a given position; and

 B: general structure of 5' donor miRNA

Fig. 3 is a set of graphs and diagrams illustrating thermodynamic stability of miRNA encoding mRNA sequence of human 3' donor:

A: thermodynamic stability profiles;

5 Upper panel: graph of the thermodynamic stability profiling at each position;

Center panel: graph of the standard deviation at each position;

10 Lower panel: graph of the number of mismatches such as an internal loop or a bulge at a given position; and

B: general structure of 3' donor miRNA

Fig. 4 is a set of graphs illustrating thermodynamic stability of miRNA encoding mRNA sequence of fly 5' donor:

15 Upper panel: graph of the thermodynamic stability profiling at each position;

Center panel: graph of the standard deviation at each position;

20 Lower panel: graph of the number of mismatches such as an internal loop or a bulge at a given position

Fig. 5 is a set of graphs illustrating thermodynamic stability of miRNA encoding mRNA sequence of fly 3' donor:

25 Upper panel: graph of the thermodynamic stability profiling at each position;

Center panel: graph of the standard deviation at each position;

Lower panel: graph of the number of mismatches such as an internal loop or a bulge at a given position

5

Fig. 6 is a concept map illustrating the minimum pri-miRNA method,

Fig. 7 is a set of diagrams illustrating the results
10 of pri-miRNA process assay with 16-TL1 and 16-TL2 variants:

A: structures of variants; and

B: pri-miRNA process assay gel

Fig. 8 is a set of diagrams illustrating the results
15 of pri-miRNA process assay with 16-TL3 variant:

A: structure of variant; and

B: pri-miRNA process assay gel

Fig. 9 is a set of diagrams illustrating the results
20 of pri-miRNA process assay with 16-TL4, 16-TL5 and 16-TL6 variants:

A: structures of variants; and

B: pri-miRNA process assay gel

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Fig. 10 is a set of diagrams illustrating the results of pri-miRNA process assay with 31-TL1 variant:

A: structure of variant; and

B: pri-miRNA process assay gel

5

Fig. 11 is a set of diagrams illustrating the results of pri-miRNA process assay with 23-TL1 and 23-TL2 variants:

A: structures of variants; and

B: pri-miRNA process assay gel

10

Fig. 12 is a set of diagrams illustrating the results of pri-miRNA process assay with 16-BS, 16-5'BS and 16-3'BS variants:

A: structures of variants; and

15

B: pri-miRNA process assay gel

Fig. 13 is a set of diagrams illustrating the results of pri-miRNA process assay with 16-BS1 and 16-BS2 variants:

A: structures of variants; and

20

B: pri-miRNA process assay gel

Fig. 14 is a set of diagrams illustrating the results of pri-miRNA process assay with 16-L-4 variant:

A: structure of variant; and

25

B: pri-miRNA process assay gel

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Fig. 15 is a set of diagrams illustrating the results of pri-miRNA process assay with 16-U+2, 16-U-2 and 16-U-6 variants:

- 5 A: structures of variants; and
 B: pri-miRNA process assay gel

Fig. 16 is a set of diagrams illustrating the results of pri-miRNA process assay with 16-TL7, 16-TL7/U-10 and 17-TL7/U-20 variants:

- 10 A: structures of variants; and
 B: pri-miRNA process assay gel

Fig. 17 is a set of diagrams illustrating the results of immunoprecipitation with Drosha-DGCR8 protein:

- 15 A: silver staining result; and
 B: UV-crosslinking result

Fig. 18 is a set of diagrams illustrating the results of immunoprecipitation with FLAG-DGCR8 protein:

- 20 A: silver staining result

Fig. 19 is a set of diagrams illustrating the results of UV crosslinking experiment using various RNAs as competitors,

25

Fig. 20 is a set of diagrams illustrating the results of UV crosslinking experiment using various RNAs as competitors:

- 5 A: result of the competition of m16-~~Δ~~BS mutant; and
 B: result of the competitions of m16-TL7, m16-TL7/U-10 and m16-TL7/U-20 mutants

10 Fig. 21 is a set of diagrams illustrating the results of pri-miRNA processing using an artificial pri-miRNA molecule:

- A: structure of the artificial pri-miRNA molecule;
 B: pri-miRNA process gel of the artificial pri-miRNA molecule; and
15 C: schematic diagram of the pri-miRNA processing

Fig. 22 is a set of diagrams illustrating the results of pri-miRNA processing using various artificial pri-miRNA molecules:

- 20 A: structures of the artificial pri-miRNA molecules;
 and
 B: pri-miRNA process gel of the artificial pri-miRNA molecule

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Fig. 23 is a diagram illustrating the results of competitions of various artificial pri-miRNAs assayed by UV crosslinking,

5 Fig. 24 is a concept map illustrating pri-miRNA processing,

Fig. 25 is a concept map illustrating AX backbone of miRNA-16:

10 A: vector map; and

B: pri-miRNA structure of AX backbone

Fig. 26 is a photograph illustrating the result of Northern blot analysis comparing the expressions between
15 wild type pri-miR-16-1 and sh backbone pri-miR-16-1,

Fig. 27 is a diagram illustrating the structure of pri-miRNA of shLuci_AX_Flat,

20 Fig. 28 is a graph illustrating the luciferase activity induced by shLuci_AX_Flat,

Fig. 29 is a diagram illustrating the structure of shLuci_AX_Flat_TL,

25

Fig. 30 is a diagram illustrating the structure of
shLuci_AX_Flat_IL,

Fig. 31 is a diagram illustrating the structure of
5 shLuci_AX_Flat_IL/B,

Fig. 32 is a diagram illustrating the structure of
shLuci_AX_Flat-30like,

10 Fig. 33 is a graph illustrating the luciferase
activity induced by shLuci_AX_Flat variant.

【Mode for Invention】

Practical and presently preferred embodiments of the
15 present invention are illustrative as shown in the
following Examples.

However, it will be appreciated that those skilled
in the art, on consideration of this disclosure, may make
modifications and improvements within the spirit and
20 scope of the present invention.

Example 1: Thermodynamic stability profiling of pri-miRNA

The present inventors collected pri-miRNA sequences
of 110 nt in length from genome sequences using genome
25 annotation information of miRBase release 7.0

(<http://microrna.sanger.ac.uk/sequences/>) which at the time of study enlisted 321 human and 68 fly miRNAs. The inventors initially prepared pri-miRNA sequences from human genome assembly NCB135 (<http://ncbi.nlm.nih.gov/Ftp/>) and pri-miRNAs from fly (*D. melanogaster*) genome assembly BDGP3 (<http://fruitfly.org/sequence/download.html>). The secondary structure of RNA was predicted using mfold program version 3.1 (<http://www.bioinfo.rpi.edu/~zukerm/rna/mfold-3.html>) (Zuker, M., *Nucleic Acids Res.* 31:3406-3415, 2003).

The present inventors then selected 280 human and 55 fly pri-miRNAs, excluding pri-miRNAs that are predicted to form multiloops, followed by thermodynamic stability profiling. The results are shown in Table 1. Human data consists of 157 and 123 pri-miRNAs respectively encoding mature mRNAs of 5' strands (5' donors) and mature mRNA of 3' strands (3' donors). In the meantime, fly data consists of 21 pri-miRNAs of 5' donors and 34 of 3' donors (Table 1). Based on the prediction of the secondary structure using mfold program, the present inventors assigned position numbers to each base-pair. Thus, the position number +1 was given to 5' end of 5'-doner originated miRNA (Fig. 2), and the position number -2 was given to 3' end of 3'-doner originated miRNA (Fig. 3).

— — — — —

Thermodynamic stability at each position in pri-miRNAs was calculated according to the nearest neighbor method using thermodynamic parameters determined at 37°C for all stacking energy values, taking into account all the different destabilizing elements such as internal loops and bulges (Mathews, D.H., et al., *J. Mol. Biol.* 288:911-940, 1999) (<http://www.bioinfo.rpi.edu/~zukerm/cgi-bin/efiles-3.0.cgi>). The method employed in this invention was previously devised by Krol and colleagues to calculate the thermodynamic features of pri-miRNAs (van der Krol et al., *Plant Cell* 2:291-299, 1990). The approach of the invention is different from that of Krol and his colleagues in the following aspects; 1) the calculation by the present inventors included -20 nt outside the miRNA hairpin whereas Krol et al. considered only the hairpin region; 2) the present inventors plotted the thermodynamic profile by calculating the ΔG values at each individual position while Krol et al. calculated the ΔG values from the windows comprising three nucleotides; 3) the present inventors included most known human and fly miRNAs in our calculation while only 13 mRNAs were calculated in Krol et al. Bulges and internal loops were considered as one position because the energy values at the bulges or internal loops cannot be divided into individual nucleotides when using the nearest neighbor model. The energy values were assigned at the

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base pair before the bulge and internal loop. Therefore, when an internal loop or bulge exists, the accurate comparison of each position becomes inevitably more difficult with increasing distance from the standard position. To avoid the positioning problem, the inventors introduced blanks at the position by the size of the bulges or internal loops and assigned the free energy value at the 3' position of the bulges or internal loops. At last, the present inventors constructed the free energy profile including blanks, which indicate unmatched pairs, insertions and deletions. The blanks were not considered when calculating the means and the standard deviations at each position.

The general structures of pri-miRNAs were predicted by averaging the free energy values at each position. The free energy values at each position were averaged and the results were shown in the graphs (human: Fig. 2 and Fig. 3; fly: Fig. 4 and Fig. 5).

Based on the values shown in the graphs, the entire structure of pri-miRNA was generalized (Fig. 2B and Fig. 3B). The corresponding sequence to that of target mRNA was searched from miR-Base, which was compared with sequences listed on <http://genome.ucsc.edu/> to determine neighbor sequences. Human and fly pri-miRNAs consist of unstable stem structures bearing three helical turns, which is

surrounded by unstable segments at both ends. Pri-miRNA is divided into 4 parts; a single strand tail, the lower stem, the upper stem harboring a target specific sequence and a terminal loop (Fig. 2 and Fig. 3). Drosha cleaved pri-miRNA at 2 helical turns away from the terminal loop and one helical turn away from the single strand tail. The upper stem was found to be stable at +3 position and free energy was higher in the middle of the upper stem (+9 ~ +12). The lower stem was 11 bp long and contained an internal loop at -6 ~ -9 position. A single tail contained a single strand or a huge bulge and/or an internal loop at irregular positions, suggesting that the energy value of the single strand tail varies.

In the case of pri-miRNAs encoding miRNA at the 5' arm, the most unstable position inside the stem corresponds to the +1 position (Fig. 2 and Fig. 4). This may explain why 5' strand is selected as a mature miRNA during miRISC assembly, as the miRNA duplex derived from these pri-miRNAs is expected to be less stable at the 5' side of the mature miRNA. Calculations for 3' donor showed relatively high free energy values at the opposite side (positions +19 ~ +20), compared with the values obtained at positions +1 ~ +2 (Fig. 3 and Fig. 5). The p-values for the differences in free energy at the +1 ~ +2 positions compared to the +19 ~ +20 positions were $5.9e-12$ and $4.2e-0.8$ for the 5' donors

and the 3' donors in humans, respectively, and 1.9×10^{-4} and 7.4×10^{-3} for the 5' donors and the 3' donors in flies, respectively. These results support that the relative instability in termini of miRNA duplex is a major determinant in strand selection.

Interestingly, the +12 position of 5' donor was comparatively unstable and the +12 position of 3' donor was also unstable (Fig. 2 and Fig. 3) (p-value: the +12 position of 5' donor: 4.1×10^{-5} ; the +9 position of 3' donor: 4.9×10^{-3}). When miRNA was 22 nt in length, the +9 position corresponded to the +12 position of 5' arm of a mature molecule. So, the +12 position of 5' arm of mature miRNA is unstable both in 5' donor and in 3' donor. Similar profile was observed in *Drosophila* miRNA (Fig. 4 and Fig. 5). From the above results, it was confirmed that thermodynamic stability at each position influences strand selection and/or other steps during RISC assembly.

【Table 1】

A. pri-miRNAs encoding miRNA at the 5' arm			
has-let-7a-2	has-mir-98	has-mir-196a-2	has-mir-452
has-let-7a-3	has-mir-99a	has-mir-196b	has-mir-485
has-let-7c	has-mir-99b	has-mir-199a-1	has-mir-488
has-let-7d	has-mir-100	has-mir-199a-2	has-mir-491
has-let-7i	has-mir-105-1	has-mir-199b	has-mir-492
has-let-7f-1	has-mir-106a	has-mir-202	has-mir-493

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has-let-7g	has-mir-106b	has-mir-24	has-mir-497
has-let-7i	has-mir-122a	has-mir-205	has-mir-498
has-mir-10a	has-mir-125a	has-mir-211	has-mir-499
has-mir-10b	has-mir-125b-1	has-mir-213	has-mir-501
has-mir-15a	has-mir-125b-2	has-mir-215	has-mir-502
has-mir-15b	has-mir-129-1	has-mir-216	has-mir-503
has-mir-16-1	has-mir-129-2	has-mir-217	has-mir-504
has-mir-16-2	has-mir-135a-1	has-mir-218-1	has-mir-510
has-mir-17	has-mir-135a-2	has-mir-218-2	has-mir-512-2
has-mir-20a	has-mir-136	has-mir-219-1	has-mir-513-1
has-mir-20b	has-mir-138-1	has-mir-219-2	has-mir-515-1
has-mir-21	has-mir-138-2	has-mir-220	has-mir-515-2
has-mir-26a-1	has-mir-139	has-mir-224	has-mir-516-3
has-mir-26a-2	has-mir-140	has-mir-296	has-mir-516-4
has-mir-26-b	has-mir-145	has-mir-299	has-mir-518a-1
has-mir-28	has-mir-146a	has-mir-302b	has-mir-518a-2
has-mir-30a	has-mir-146b	has-mir-325	has-mir-518c
has-mir-30b	has-mir-149	has-mir-335	has-mir-518d
has-mir-30c-1	has-mir-150	has-mir-339	has-mir-518e
has-mir-30d	has-mir-155	has-mir-345	has-mir-518f
has-mir-31	has-mir-181a	has-mir-361	has-mir-519b
has-mir-32	has-mir-181c	has-mir-362	has-mir-519c
has-mir-33	has-mir-181d	has-mir-369	has-mir-519e
has-mir-34a	has-mir-182	has-mir-374	has-mir-520d
has-mir-34b	has-mir-183	has-mir-379	has-mir-522
has-mir-24c	has-mir-185	has-mir-382	has-mir-523
has-mir-7-1	has-mir-186	has-mir-383	has-mir-524
has-mir-7-2	has-mir-188	has-mir-422a	has-mir-526a-1
has-mir-7-3	has-mir-18a	has-mir-431	has-mir-526a-2

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has-mir-9-1	has-mir-18b	has-mir-432	has-mir-526b
has-mir-9-2	has-mir-192	has-mir-449	has-mir-527
has-mir-93	has-mir-194-1	has-mir-450-1	
has-mir-9-3	has-mir-195	has-mir-450-2	
has-mir-96	has-mir-196a-1	has-mir-451	
B. pri-miRNAs encoding miRNA at the 3' arm			
has-mir-1-2	has-mir-133a-2	has-mir-302d	has-mir-490
has-mir-19a	has-mir-133b	has-mir-320	has-mir-495
has-mir-19b-1	has-mir-137	has-mir-323	has-mir-496
has-mir-19b-2	has-mir-141	has-mir-326	has-mir-500
has-mir-22	has-mir-142	has-mir-328	has-mir-505
has-mir-23a	has-mir-143	has-mir-329-2	has-mir-506
has-mir-23b	has-mir-144	has-mir-330	has-mir-507
has-mir-24-2	has-mir-148a	has-mir-331	has-mir-508
has-mir-27a	has-mir-148b	has-mir-338	has-mir-509
has-mir-27b	has-mir-152	has-mir-340	has-mir-514-1
has-mir-29a	has-mir-153-2	has-mir-342	has-mir-514-2
has-mir-29b-2	has-mir-154	has-mir-363	has-mir-514-3
has-mir-29c	has-mir-184	has-mir-367	has-mir-516-1
has-mir-30e	has-mir-187	has-mir-368	has-mir-516-2
has-mir-92-1	has-mir-191	has-mir-370	has-mir-517a
has-mir-95	has-mir-193a	has-mir-371	has-mir-517b
has-mir-101-1	has-mir-193v	has-mir-372	has-mir-517c
has-mir-101-2	has-mir-197	has-mir-373	has-mir-518b
has-mir-103-1	has-mir-200b	has-mir-375	has-mir-519a-1
has-mir-103-2	has-mir-200c	has-mir-376a	has-mir-519a-2
has-mir-107	has-mir-203	has-mir-376b	has-mir-519d
has-mir-124a-1	has-mir-206	has-mir-377	has-mir-520a
has-mir-124a-2	has-mir-208	has-mir-378	has-mir-520c

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has-mir-124a-3	has-mir-210	has-mir-381	has-mir-520e
has-mir-127	has-mir-214	has-mir-409	has-mir-520f
has-mir-128a	has-mir-221	has-mir-410	has-mir-520g
has-mir-128b	has-mir-222	has-mir-412	has-mir-520h
has-mir-130a	has-mir-223	has-mir-413	has-mir-521-1
has-mir-130b	has-mir-301	has-mir-433	has-mir-521-2
has-mir-132	has-mir-302a	has-mir-448	has-mir-525
has-mir-133a-1	has-mir-302c	has-mir-489	
C. fly pri-miRNAs encoding miRNA at the 5' arm			
dme-let-7	dme-mir-11	dme-mir-263a	dme-mir-289
dme-mir-5	dme-mir-13a	dme-mir-263b	dme-mir-305
dme-mir-7	dme-mir-13b-1	dme-mir-274	dme-mir-315
dme-mir-9a	dme-mir-34	dme-mir-276a	
dme-mir-9b	dme-mir-125	dme-mir-276b	
dme-mir-9c	dme-mir-219	dme-mir-292	
D. fly pri-miRNAs encoding miRNA at the 3' arm			
dme-bantam	dme-mir-6-2	dme-mir-275	dme-mir-209
dme-mir-iab-4	dme-mir-6-3	dme-mir-277	dme-mir-310
dme-mir-1	dme-mir-8	dme-mir-278	dme-mir-311
dme-mir-2a-1	dme-mir-14	dme-mir-279	dme-mir-312
dme-mir-2a-2	dme-mir-79	dme-mir-281-1	dme-mir-313
dme-mir-2b-2	dme-mir-87	dme-mir-281-2	dme-mir-317
dme-mir-3	dme-mir-92a	dme-mir-285	dme-mir-318
dme-mir-4	dme-mir-124	dme-mir-288	
dme-mir-6-1	dme-mir-184	dme-mir-306	

Example 2: Purification of the recombinant DGCR8 protein

HEK293T cells (ATCC, USA) were transfected with FLAG-DGCR8 expression vector, followed by purification with the

method of Han et al. (Han et al., *GENES & DEVELOPMENT*, 3016-3027, 2004). Two days after transfection, the transformed HEK293T cells were harvested and sonicated in ice-cold buffer D-K'200 (20 mM Tris, pH 8.0, 200 mM KCl, 0.2 mM EDTA, and 0.2 mM PMSF). After centrifugation at 13,200 rpm at 4°C for 15 minutes, the supernatant was treated with 50 µg/ml of RNase A at 4°C for 30 minutes. The extract was then incubated with anti-FLAG antibody (Sigma, USA) conjugated agarose beads (Sigma, USA) with constant rotation at 4°C for 120 minutes. The beads were washed four times with N-Da'2500 buffer (20 mM Tris, pH 8.0, 2.5 M NaCl, 0.2 mM EDTA, 0.2 mM PMSF, 1% Triton X-100) and then three times with FLAG-elution buffer (50 mM Tris, pH 7.4, 150 mM NaCl). The protein was eluted with FLAG-elution buffer containing 400 µg/ml of 3X FLAG peptide (Sigma, USA) at 4°C for 60 minutes and then concentrated into 20 ng/ml by using Centricon YM-30 (Millipore, USA).

Example 3: Mutagenesis and *in vitro* processing assay

Based on the results of Example 1, mutations were introduced into each part of pri-miRNAs to examine their significance in pri-miRNA processing and the results are shown in Table 2. Because miRNA maturation is a multi-step process *in vivo*, certain mutations may affect not only the pri-miRNA processing step but also other steps such as pri-

miRNA export, cytoplasmic processing and RNA turnover. To avoid such complications, the present inventors assayed the pri-miRNA cleavage reaction *in vitro* using labeled transcripts and an immunopurified Drosha-DGCR8 complex.

5 Mutagenesis was carried out based on the "minimal pri-miRNAs" that was previously developed by the present inventors (Han, J., *et al.*, *Genes Dev.* 18:3016-3027, 2004) (Fig. 6). Minimal pri-miRNAs contain pri-miRNA sequences plus -20 nt sequences outside of the Drosha cleavage sites.
10 For efficient transcription by T7 RNA polymerase, two additional Gs were incorporated between the promoter and the pri-miRNA sequences (Fig. 6).

An *in vitro* processing assay was carried out by incubating RNA with immunoprecipitated FLAG-tagged Drosha.
15 The Drosha-DGCR8 complex (Microprocessor) cleaves pri-miRNAs, yielding three kinds of fragments which are the 5' flanking fragment (F1, -25 nt), pri-miRNA (F2, -65 nt) and the 3' flanking fragment (F3, -20 nt) (Fig. 6). To identify these fragments, processing reactions were carried
20 out using 5' end-labeled RNA as well as internally labeled RNA. When necessary, the cleavage products were gel-purified, ligated to 3' and 5' adapters, reverse-transcribed, PCR amplified, inserted into pGEM-T easy vector and confirmed by sequencing. Alternatively, some

fragments were gel-purified and analyzed by primer extension.

【Table 2】

Mutant	Mutation
miR-16-TL1	terminal loop converted into single strand (Fig. 7)
miR-16-TL2	sequence extended in the sliced loop of miR-16-TL1 (Fig. 7)
miR-16-TL3	basal segments and terminal loop converted into internal loop at both ends to form double strand (Fig. 8)
miR-16-TL4	basal segments and terminal loop substituted to generate inverted hairpin (Fig. 9)
miR-16-TL5	single strand extended in inverted hairpin (Fig. 9)
miR-16-TL6	single strand converted into double strand in inverted hairpin (Fig. 9)
miR-31-TL1	basal segments and terminal loop substituted to generate inverted hairpin (Fig. 10)
miR-23-TL1	basal segments and terminal loop substituted to generate inverted hairpin (Fig. 11)
miR-23-TL2	single strand converted into double strand in inverted hairpin (Fig. 11)
miR-16-△BS	single strand eliminated (Fig. 12)
miR-16-5'BS	3' end of single strand eliminated (Fig. 12)
miR-16-3'BS	5' end of single strand eliminated (Fig. 12)
miR-16-BS1	sequence altered in single strand (Fig. 13)
miR-16-BS2	single strand converted into double strand (Fig. 13)
miR-16-L-4	4 bp eliminated in the lower stem (Fig. 14)
miR-16-U+2	2 bp added in the upper stem (Fig. 15)
miR-16-U-2	2 bp eliminated in the upper stem (Fig. 15)
miR-16-U-6	6 bp eliminated in the upper stem (Fig. 15)
miR-16-TL7	terminal loop reduced (Fig. 16)
miR-16-TL7/U-10	10 bp reduced in the upper stem and terminal loop (Fig. 16)
miR-16-TL7/U-	20 bp reduced in the upper stem and terminal

20	loop (Fig. 16)
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<3-1> Preparation of double stranded RNA

To construct pri-miRNA harboring a terminal loop, each strand of RNA was transcribed *in vitro*. A template for the transcription was prepared by PCR using the following primers. Particularly, pri-miR-16 gene operably linked to T7 promoter was inserted into p-GEM-T easy vector (Promega, USA), which was then named "pNKA57" and used as a template. To produce strand A of 16-TL1, 16-WT-F primer represented by SEQ. ID. NO: 1 (5'-TAA TAC GAC TCA CTA TAG GTG ATA GCA ATG TCA GCA GTG-3') and a reverse primer represented by SEQ. ID. NO: 2 (5'-AGA ATC TTA ACG CCA ATA TTT AC-3') were used. To produce strand B of 16-TL-1, 16-WT-R primer represented by SEQ. ID. NO: 3 (5'-GTA GAG TAT GGT CAA CCT TA-3') and a forward primer represented by SEQ. ID. NO: 4 (5'-TAA TAC GAC TAC CTA TAG GAA AAT TAT CTC CAG TAT TAA C-3') were used. To produce strand A of 16-TL-2, 16-WT-F primer and a reverse primer represented by SEQ. ID. NO: 5 (5'-ACA GTA TAC GCC AAT ATT TAC GTG C-3') were used. To produce strand B of 16-TL-2, the same primers used for the production of strand B of 16-TL-1 were used. To prepare 16-TL-3, miR-15-a ~ 16-1 partial gene was inserted into pGEM-T-easy vector (Promega, USA), which was then used as a template. To produce strand A of 16-TL3, a primer set

each represented by SEQ. ID. NO: 6 (5'-TAA TAC GAC TCA CTA TAG GAG CTC TTA TGA TAG CAA TGT C-3') and SEQ. ID. NO: 7 (5'-GGA ACG TTA ATT TTA GAA TCT TAA CGC CAA TAT TTA C-3') were used. To produce strand B of 16-TK3, a primer set each represented by SEQ. ID. NO: 8 (5'-TAA TAC GAC TCA CTA TAG GAA AAT TAT CTC CAG TAT TAA C-3') and SEQ. ID. NO: 9 (5'-GTA GAG TAT GGT CAA CCT TA-3') were used. RNA transcripts were heated at 95°C for 15 seconds in annealing buffer (Tris pH 7.5 10 mM, EDTA pH8.0 10 mM/volume: 20 μ l ~ 50 μ l according to the amount of RNA), then cooled down slowly, resulting in miR-16-TL1, TL2 and TL3 (Fig. 7, Fig. 8 and Table 3). Nonirradiated transcripts were processed with immunoprecipitated Drosha-FLAG at 37°C for 60 minutes as follows. Pri-miRNA was *in vitro* processed using the produced miRNA according to the method of Lee, et al (Lee et al., *Nature* 425:415-419, 2003; Lee et al., *EMBO J.* 21:4663-4670, 2002). 30 μ l of the reaction solution contained 6.4 mM MgCl₂, 1 unit/ μ l of Ribonuclease inhibitor (Takara, Japan), $1 \times 10^4 \sim 1 \times 10^5$ cpm transcripts and 15 μ l of immunoprecipitated buffer D' beads. The reaction mixture was then processed at 37°C for 60 minutes. RNA was phenol-extracted from the reaction mixture, followed by electrophoresis on 12.5% denaturing urea-polyacrylamide gel. RNA size markers (Decade marker, Ambion) labeled at the 5'

end were used. The synthetic RNA of 23 nt and 27 nt was labeled at the 5' end and used as an additional marker.

The fragment of interest was gel-purified and ligated to the 3' adaptor. The ligated product was gel-purified and ligated to the 5' adaptor. The 3' and 5' adaptors used for the cloning were 5'-pUU Uaa ccg cga att cca gidT-3' (uppercase: RNA; lower case: DNA; p: phosphate; idT: deoxythymidine) and 5'-acg gaa ttc ctc act AAA-3' (uppercase: RNA; lowercase: DNA). Reverse transcription was performed using a reverse transcription primer represented by SEQ. ID. NO: 10 (5'-ACT GGA ATT CGC GGT TAA A-3'). Then, a forward primer represented by SEQ. ID. NO: 11 (5'-CAG CCA ACG GAA TTC CTC CTC ACT AAA-3') and a reverse primer represented by SEQ. ID. NO: 12 (5'-GGA ATT CGC GGT TAA A-3') were utilized for PCR amplification. PCR was performed as follows; predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, polymerization at 72°C for 30 seconds, 30 cycles from denaturation to polymerization, and final extension at 72°C for 7 minutes. The PCR product was subcloned into pGEM-T-easy vector (Promega, USA) and 10 clones were sequenced at Genome Analysis Unit of Seoul National University (Seoul, Korea).

To investigate the role of a terminal loop in pri-miRNA processing, the present inventors eliminated the

terminal loop by converting it into two separate ssRNA segments (16-TL1) prior to *in vitro* pri-miRNA processing. The variant 16-TL1 lacking a terminal loop was processed at the original site (Fig. 7). A similar mutant, 16-TL2, containing extended sequence in the sliced loop was also processed at the original site (Fig. 7). Another mutant 16-TL3 with the substitution of a terminal loop with a larger loop contained extended stems at both ends (Fig. 8). The variant 16-TL3 was also processed accurately. The above results indicate that the terminal loop structure itself is not necessary for pri-miRNA processing. The Drosha-DGCR8 complex may therefore process not only hairpin RNAs, but also other substrates such as long dsRNAs with large internal loops.

<3-2> Construction of hairpin variants

The present inventors prepared pri-miRNA with inverted hairpin structure having alterations in the basal segments wherein a single strand segments were linked to a terminal loop and the terminal loop was linked to a single strand segment area. The two strands of the variant of this example were transcribed as described in Example <3-1> and ligated using T4 DNA ligase and DNA bridge. The DNA bridge bears corresponding sequences to miR-16-1 single strand segments represented by SEQ. ID. NO: 13 (5'-CAT TGC

TAT CAC CGT AGA GTA TGG-3'). Strand A of pri-miR-16-TL-1 was dephosphorylated by calf intestine phosphatase (Takara, Japan). For the dephosphorylation, RNA, 1 μ l of dephosphatase and 2 μ l of dephosphorylation buffer were mixed and the total volume was adjusted to 20 μ l by adding distilled water, followed by reaction at 37°C for one hour. Strands A and B of pri-miRNA-16 and DNA bridge were mixed together (RNA strand A:RNA strand B = 1:1, DNA bridge: 0.375 pmol), to which 350 U of T4 DNA ligase (Takara, Japan) was added, followed by reaction at 30°C for 4 hours. RNA was phenol-extracted from the reaction mixture and electrophoresed on 6% denaturing urea-polyacrylamide gel. RNA was extracted from the gel by cutting the migrated band. The ligated RNA was used for reverse transcription using SUPERScript II (Invitrogen, USA). A reverse primer represented by SEQ. ID. NO: 2 was used for the reverse transcription, which was the one that used for the amplification of strand A of 16-TL1. Templates of 16-TL4, 16-TL5 and 16-TL6 were prepared using those templates listed in Table 3 by the same manner as described in Example <3-1>. To amplify the template of 16-TL4, forward primers represented by SEQ. ID. NO: 14 (5'-GTA GAG TAT GGT CAA CCT TA-3') used for the production of strand B of TL1 and represented by SEQ. ID. NO: 4 were used. Following primers were used for PCR with 16-TL5 and 16-TL6. Forward

primers that were used for the strand B of each TL1 and TL2 were used. And a primer represented by SEQ. ID. NO: 5 and a primer represented by SEQ. ID. NO: 15b(5'-AGA AAA TTA TCT CCA ATA TTT ACG TGC TGC-3') were used as reverse primers.

5 PCR was performed as follows; predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, polymerization at 72°C for 30 seconds, 20 cycles from denaturation to polymerization, and final extension at 72°C for 7 minutes. To produce 31-TL1, 23-TL1

10 and 23-TL2, two oligonucleotides were prepared for each. Templates of 31-TL1, 23-TL1 and 23-TL2 were prepared using the templates listed in Table 3 by the same manner as described in Example <3-1>. 31-TL1 oligomer was amplified

15 using the oligomer represented by SEQ. ID. NO: 16 (5'-GGG AAC CTG CTA TGC CAA CAT ATT GCC ATC TTT CCT GTC TGA CAG ACT TGG AAC TG-3') and the oligomer represented by SEQ. ID. NO: 17 (5'-ATG TCA ACA GCT ATG CCA GCA TCT TGC CTC CTC TCC AGT TCC AAG TCT GTC AGA C-3') by expanded high fidelity PCR

20 system (Roche, USA). 150 pmol of each primer, 6 μ l of dNTPmix, 1 μ l of enzyme, 10 μ l of 5X buffer and 30 μ l of distilled water were mixed, followed by denaturation at 94°C for three minutes. Then, reaction was continued at 40°C for one minute, followed by extension at 72°C for 10 minutes.

The reaction product was amplified by PCR using the
25 following primers; a forward primer represented by SEQ. ID.

NO: 18 (5'-TAA TAC GAC TCA CTA TAG GGA ACC TGC TAT GCC AAC-3') and a reverse primer represented by SEQ. ID. NO: 19 (5'-CTG GCA TAG CTG TTG AAC T-3'). PCR was performed as follows; predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, polymerization at 72°C for 30 seconds, 25 cycles from denaturation to polymerization, and final extension at 72°C for 7 minutes. 23-TL1 and 23-TL2 were amplified using oligomers each represented by SEQ. ID. NO: 20 (5'-GTG TCA CAA ATC ACA TTG CCA GGG ATT TCC AAC CGA CCC TGA GCT CTG CCT GTG CCA C-3') and SEQ. ID. NO: 21 (5'-GGA AGC AAA TTC CAT CCC CAG GGA ACC CCA GCC GGC CGT GGC ACA GGC AGA GCT CAG-3') by expanded high fidelity PCR system (Roche, USA). 150 pmol of each primer, 6 μ l of dNTPmix, 1 μ l of enzyme, 10 μ l of 5X buffer and 30 μ l of distilled water were mixed, followed by denaturation at 94°C for 3 minutes and the reaction continued at 40°C for one minute, followed by extension at 72°C for 10 minutes. The reaction products were amplified by PCR as follows; 23-TL1 was amplified using a forward primer represented by SEQ. ID. NO: 22 (5'-TAA TAC GAC TCA CTA TAG GTG TCA CAA ATC ACA TTG C-3') and a reverse primer represented by SEQ. ID. NO: 23 (5'-GGA AGC AAA TCC CAT CCC CAG-3'). PCR was performed as follows; predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds,

polymerization at 72°C for 30 seconds, 25 cycles from denaturation to polymerization, and final extension at 72°C for 7 minutes. To amplify 23-TL2, the forward primer that was used for the amplification of 23-TL1 was used together
5 with a reverse primer represented by SEQ. ID. NO: 24 (5'-GGT GTC ACA AAT CCC ATC CCC AGG A-3'). PCR was performed as follows; predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, polymerization at 72°C for 30 seconds, 25 cycles
10 from denaturation to polymerization, and final extension at 72°C for 7 minutes. As a result, pri-miRNA 16-TL4, TL-5 and TL-6 were prepared (Table 3). Those products were *in vitro* processed by the same manner as described in Example <3-1>.

The inverted hairpin variant 16-TL4 was processed at
15 the original site though with less accuracy and efficiency (Fig. 9; lines 6 and 10). When the sequences in the cleaved terminal loop were modified to create less stable ssRNA region (16-TL5), this variant was processed more efficiently at the precise cleavage site (Fig. 9; lines 7
20 and 11). The additional inverted hairpin variant (16-TL6) containing an extended stem was cleaved similarly to 16-TL4, at the original site (Fig. 9; lines 8 and 12). The above results suggest that terminal loop structure itself is not important for cleavage site selection in pri-miRNA-16-1.
25 The present inventors generated two more hairpin variants

31-TL1 and 23-TL1 based on pri-miR-31 and pri-miR-23 by the method described above (Fig. 10 and Fig. 11). These variants were processed with Drosha-DGCR8 complex as shown in Example <3-1>. As a result, they were cleaved efficiently at the original sites (Fig. 10 and Fig. 11). Another miR-23 variant 23-TL2 was also cleaved efficiently at the original site (Fig. 11). The results also indicate that the terminal loop is not imperative for cleavage site selection.

<3-3> Construction of single strand tail variants

Under the conditions of Table 3, pri-miRNA 16- Δ BS, 5'BS, 3'BS, BS-1 and BS-2 molecules were prepared by the same manner as described in Example <3-2>. These produced molecules were processed *in vitro* by the same manner as described in Example <3-1>.

To investigate molecular basis of pri-miRNA processing, the present inventors generated a series of mutations with alterations in single strand tail (Fig. 12). When a single strand tail was eliminated from pri-miR-16-1, *in vivo* pri-miRNA processing was abolished (Fig. 12, 16- Δ BS). Mutants retaining only one side of the flanking strands (16-5'BS, 16-3'BS) were processed although the efficiency was compromised (Fig. 12), suggesting that only

one side of flanking strands (either 5' or 3' basal segment) could support processing.

To further examine the contribution of the basal segments to processing, the present inventors altered their sequences. The mutant molecule 16-BS1 that retains its single-stranded structure in the basal segment region was processed efficiently (Fig. 13). But, when the single-strands of the basal segments were converted into a double strand (16-BS2), the cleavage reaction was blocked (Fig. 13). Thus, it was confirmed that it is the single-stranded nature of the basal segments, rather than the nucleotide sequences that may be critical for Drosha processing.

<3-4> Generation of mutants with alteration of the distance from single strand tail

Under the conditions of Table 3 and Table 4, pri-miRNA 16-L-4, 16-U-2, 16-U-6, 16-TL7, 16-TL7/U-10 and 17-TL7/U-20 were prepared by the same manner as described in Example <3-2>.

Mammalian pri-miRNAs typically contain 3 helical turns in a stem. Zeng et al. previously suggested that the sites of Drosha cleavage may be determined largely by the distance (-22 nt) from the terminal loop (Zeng, Y., et al., *EMBO J* 21:5875-5885, 2005). In their study, the authors were able to show that when a pri-miR-30a variant was

modified such that the loop-stem junction moves either 1 bp up the stem or 1 bp down the stem, the cleavage site shifted either 1 bp up the stem or 1 bp down the stem, respectively. The present inventors have independently performed extensive experiments to address this same issue but our data are in fact inconsistent with this "loop-stem junction anchoring" model.

The present inventors introduced a deletion into the lower stem of the pri-miR-16-1 to reduce the distance from the vassal segments. In a mutant where the distance from the basal segments decreases by 4 bp (16-L-4), the cleavage site was shifted by 4 bp away from these segments (Fig. 14). The inventors then altered the distance between the cleavage site and the terminal loop by deletion or insertion in the upper stem of pri-miR-16-1 (Fig. 15). Three upper-stem mutants were found to have been cleaved at the original site, in spite of such deletions or insertions (16-U+2, 16-U-2 and 16-U-6). A mutant containing a smaller terminal loop was also cleaved at the same site (Fig. 15). The inventors also introduced deletions into the upper stem to generate variants with a small terminal loop and a shorter stem (16-TL7/U-10 and 16-TL7/U-20) and observed that these substrates were also cleaved at the original sites albeit at a lower efficiency (Fig. 16). As shown in Example <3-1>, the mutant lacking a terminal loop was

cleaved at the original site (Fig. 7). From the above results, it was confirmed that the distance from the terminal loop is unlikely to be the major determinant of cleavage site selection.

5 However, the processing efficiency of the small terminal loop mutant (16-TL7) was slightly affected (Fig. 16), suggesting that a flexible terminal loop may be beneficial in the reaction. Also, the processing became less efficient when there were reduced lengths of the stem
10 (16-TL7/U-10 and 16-TL7/U20), suggesting that the microprocessor may need to contact the whole length (33bp) of the hairpin for full activity.

【Table 3】

Mutant	Primer sequence (5' → 3') (SEQ. ID. NO)	Substrate	PCR condition
miR-16- TL1 A	S:16-WT- F(TAATACGACTCACTATAG GTGATAGCAATGTCAGCAGT G) (1) AS: (AGAATCTTAACGCCAA TATTTAC) (2)	pNKA-57	predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, polymerization at 72°C for 30 seconds, 20 cycles from denaturation to polymerization, and final extension at 72°C for 7 minutes
miR-16- TL1 B	S:TAATACGACTACCTATAG GAAAATTATCTCCAGTATTA AC (4)	pNKA-57	Same as above

	AS:16-WT- R (GTAGAGTATGGTCAACCT TA) (3)		
miR-16- TL2 A	S:16-WT-F(1) AS:ACAGTATACGCCAATAT TTACGTGC) (5)	pNKA-57	Same as above
miR-16- TL2 B	S:TAATACGACTACCTATAG GAAAATTATCTCCAGTATTA AC) (4) AS:16-WT- R (GTAGAGTATGGTCAACCT TA) (3)	pNKA-57	Same as above
miR-16- TL3 A	S:TAATACGACTCACTATAG GAGCTCTTATGATAGCAATG TC) (6) AS:GGAACGTTAATTTTGA ATCTTAACGCCAATATTTAC) (7)	pNKA- 3 (miR- 15a~16- 1pGEM- Teasy)	Same as above
miR-16- TL3 B	S:TAATACGACTCACTATAG GAAAATTATCTCCAGTATTA AC) (8) AS:GTAGAGTATGGTCAACC TTA) (9)	pNKA-3	Same as above
miR-16- TL4	S:TAATACGACTACCTATAG GAAAATTATCTCCAGTATTA AC) (4) AS:(AGAATCTTAACGCCAA TATTTAC) (14)	pNKA- 145 (pri- miR-16- 1TL4pGEM- Teasy)	Same as above
miR-16- TL5	S:TAATACGACTACCTATAG GAAAATTATCTCCAGTATTA AC) (4) AS:ACAGTATACGCCAATAT TTACGTGC) (5)	pNKA-145	Same as above
miR-16- TL6	S:TAATACGACTACCTATAG GAAAATTATCTCCAGTATTA AC) (4) AS:- AGAAAATTATCTCCAATATT TACGTGCTGC) (15)	pNKA-145	Same as above
miR-31- TL1	S:(TAATACGACTCACTATA GGGAACCTGCTATGCCAAC) (18) AS:(CTGGCATAGCTGTTGA ACT) (19)	pNKA- 146 (pri- miR- 31TL1pGEM- Teasy)	Same as above
miR-23-	S:TAATACGACTCACTATAG	pNKA-	Same as above

TL1	GTGTCACAAATCACATTGC) (22) AS: (GGAAGCAAATCCCATC CCCAG (23)	147 (pri- miR- 23aTL1pGEM -Teasy)	
miR-23- TL2	S:TAATACGACTCACTATAG GTGTCACAAATCACATTGC) (22) AS:GGTGTACAAATCCCAT CCCCAGGA) (24)	pNKA-147	Same as above
miR-16- BS	S:BS- F(TAATACGACTCACTATAG GTCAGCAGTGCCTTAGCAGC AC) (25) AS:BS- R(GTCAACCTTACTTCAGCA GC) (26)	pNKA-57	Same as above
miR-16- 5'BS	S:16-WT-F(1) AS:BS-R(26)	pNKA-57	Same as above
miR-16- 3'BS	S:BS-F(25) AS:16-WT-R(3)	pNKA-57	Same as above
miR-16- BS1	S:BS1- F(TAATACGACTCACTATAG GCTTAAAATAAGTCAGCAGT GCCTTAGCAG) (27) AS:BS1- R(CATACATCGTGTCAACCT TACTTCAGCAG) (28)	pNKA-57	Same as above
miR-16- BS2	S:BS1-F(27) AS:BS2- R(GGCTTAAAATAAGCTAAC CTTACTTCAGCAG) (29)	pNKA-57	Same as above
miR-16- L-4	S:L-4- F(TAATACGACTCACTATAG GTGATAGCAATGAGCTGCCT TAGCAGC) (30) AS:L-4- R(GTAGAGTATGGAACACT TCAGC) (31)	pNKA-57	Same as above
miR-16- U+2	S:16-WT-F(1) AS:16-WT-R(3)	pNKA- 55 (pri- miR-16- 1U+2pGEM- Teasy)	Same as above
miR-16- U-2	S:16-WT-F(1) AS:16-WT-R(3)	pNKA- 72 (pri-	Same as above

		miR-16-1U-2pGEM-Teasy)	
miR-16-U-6	S:16-WT-F(1) AS:16-WT-R(3)	pNKA-76(pri-miR-16-1U-6pGEM-Teasy)	Same as above
miR-16-TL7	S:WT-TL7-F(TAATACGACTCACTATAGGTGATAGCAATGTCAGCAGTGCCTTAGCAG)(32) AS:WT-TL7-R(GTAGAGTATGGTCAACCTTACTTCAGCAG)(33)	pNKA-260(pri-miR-16-1TL7pGEM-Teasy]	predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 42°C for 30 seconds, polymerization at 72°C for 30 seconds, 20 cycles from denaturation to polymerization, and final extension at 72°C for 7 minutes
miR-16-TL7/U-10	S:16-WT-F(1) AS:16-WT-R(3)	pNKA-261(pri-miR-16-1TL7/U-10pGEM-Teasy)	Same as above
miR-16-TL7/U-20	S:16-WT-F(1) AS:16-WT-R(3)	pNKA-262(pri-miR-16-1TL7/U-20pGEM-Teasy)	Same as above

*S: Forward primer

AS: Reverse primer

【Table 4】

Mutant		Oligonucleotide sequence (5'→3')(SEQ. ID. NO)
T7pri-miR-16-1U+2	Oligo 1	TGATAGCAATGTCAGCAGTGCCTTAGCAGCACGT AAATATTGTGGCGTTAAGATTCTAAAATTATC (34)

	Oligo 2	GTAGAGTATGGTCAACCTTACTTCAGCAGCACAG TTAATACTGTGGAGATAATTTTAGAATCTTAAC (35)
T7pri-miR- 16-1U-2	Oligo 1	TAATACGACTCACTATAGGTGATAGCAATGTCAGC AGTGCCTTAGCAGCACGTAAATAGGCGTTAAGATTC (36)
	Oligo 2	GTAGAGTATGGTCAACCTTACTTCAGCAGCACAG TTAATAGGAGATAATTTTAGAATCTTAACGCCTATT (37)
T7pri-miR- 16-1U-6	Oligo 1	TAATACGACTCACTATAGGTGATAGCAATGT CAGCAGTGCCTTAGCAGCACGTAAATATTGGGATTC (38)
	Oligo 2	GTAGAGTATGGTCAACCTTACTTCAGCAGCAC AGTTAATACTGGTTTTAGAATCCCAATATTTACGT (39)
T7pri-miR- 16-TL7	Oligo 1	GTCAGCAGTGCCTTAGCAGCACGTAAA TATTGGCGTTAAGATTCTGAATCT (40)
	Oligo 2	GTCAACCTTACTTCAGCAGCACAGTTAA TACTGGCGTTAAGATTCTGAATCTTAA (41)
T7pri-miR- 16-TL7/U- 10	Oligo 1	GTCAGCAGTGCCTTAGCAGCACG TAAATAGATTCTGAATCTAT (42)
	Oligo 2	GTCAACCTTACTTCAGCAGCACAG TTAATAGATTCTGAATCTA (43)
T7pri-miR- 16-TL7/U- 20	Oligo 1	GTCAGCAGTGCCTTAGCAG CGATTCTGAATCGC (44)
	Oligo 2	GTCAACCTTACTTCAGCAGC GATTCTGAATCGCT (45)

Example 4: UV crosslinking experiment

The Drosha-DGCR8 complex contains at least three dsRBDs, one on Drosha and two on DGCR8 but no known ssRNA-binding domain has been identified in either of these proteins. Although partial fragments of Drosha and DGCR8 proteins have previously been shown to bind to ssRNA and dsRNA in simple GST-pull down experiments, the relative affinities of these proteins to various RNA species have not been determined (Zeng, Y., et al., *EMBO J* 21:5875-5885, 2005).

Drosha-DGCR8 protein was fused to the FLAG epitope (FLAG-DGCR8, Drosha-FLAG) and then co-expressed and immunopurified from HEK293T cells (Fig. 17A). HEK293T cells were incubated in DMEM medium (WelGENE, Korea) containing 10% FBS (WelGENE, Korea). The HEK293T cells were then transfected with 8 μ g of pCK-Drosha-FLAG and/or 5 μ g of pCK-FLAG-DGCR8 by calcium phosphate method. pCK-Drosha-FLAG and pCK-FLAG-DGCR8 were prepared by the method of Lee et al or Han, et al (Lee et al., *Nature* 415-419, 2003; Han et al., *GENES & DEVELOPMENT*, 3016-3027, 2004). Subsequent silver staining indicated that full length DGCR8, full length Drosha and two truncated forms of Drosha were purified (Fig. 17A). To investigate which factor of microprocessor directly interacts with pri-miRNA, radio-labeled pri-miRNA-16-1 was reacted with Drosha and DGCR8, followed by UV-cross-linking experiments. Particularly, 20 ~ 50 ng of FLAG-DGCR8, 15 μ l of 1×10^6 cpm radio-labeled RNA (50~100 pmole), binding buffer (10 mM Tris, pH 7.5 KCl, 0.5 mM DTT) and 1 U of RNase (Takara, Japan) were mixed in a 96 well plate, followed by reaction at 4°C for 30 minutes. The RNAs were either prepared *in vitro* transcription (pri-miR-16-1, m16-~~BS~~, m16-TL1, pri-miR-30a, and 80 bp dsDNA) or purchased from Samchully Pharmaceuticals (siRNA duplex, 23 nt ssRNA, and pre-miR-30a). The 96 well plate containing the reaction mixture was brought into contact with a UV-

lamp in a UV cross-linker (CL-1000 UV-crosslinker, UVP) for 5 minutes. The mixture was treated with the RNase A/T1 mixture and subsequently loaded on 7.5% SDS-PAGE gel, followed by electrophoresis. By the above manner, after
5 treating RNase A/T1 after UV cross-linking, DGCR8 protein was detected by observing radio-activity (Fig. 17B). As a result, the inventors were unable to detect any significant RNA binding activity of Drosha, which suggests that Drosha may interact with its substrate only transiently during the
10 catalytic reaction, whereas DGCR8 associates directly with the substrate in a more stable manner.

To further examine the mode of interaction between DGCR8 and RNA, the FLAG-DGCR8 protein product was immunoprecipitated and washed intensively with high salt
15 buffer (2.5 M NaCl 20 mM, Tris pH8.0, 0.2 mM, EDTA pH8.0) (Figure 18). Various RNA molecules used for UV cross-linking experiments are as follows. To generate forward and reverse transcripts of 80 bp long double stranded RNA, firefly luciferase cDNA was amplified by PCR using pGL3
20 vector (Promega, USA) as a template. The forward primer used for the PCR contained T7 promoter sequence at the 5' end. A forward primer for sense strand represented by SEQ. ID. NO: 46 (5'- TTA ATA CGA CTC ACT ATA GGG CAT TTC GCA GCC TAC CGT GG-3') and a reverse primer for sense strand
25 represented by SEQ. ID. NO: 47 (5'-TTG GGA GCT TTT TTT GCA

CGT TC-3'), a forward primer for antisense strand represented by SEQ. ID. NO: 48 (5'-TTA ATA CGA CTC ACT ATA GGG AGC TTT TTT TGC ACG TTC AA-3') and a reverse primer for antisense strand represented by SEQ. ID. NO: 49 (5'-ATG GGC ATT TCG CAG CCT ACC G-3') were used. PCR was performed as follows; predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, polymerization at 72°C for 30 seconds, 25 cycles from denaturation to polymerization, and final extension at 72°C for 7 minutes. The PCR product was utilized as a template for *in vitro* transcription to produce dsRNA sense and antisense transcripts. The sense-antisense pair was reacted at 30°C for one hour in 1X universal buffer (6 mM HEPES-KOH, pH 7.5, 20 mM KCl, 0.2 mM MgCl₂), followed by boiling at 90°C for 2 minutes for annealing to duplex. The sequences of 23 nt dsRNA and siRNA duplex are as follows; 23 nt single stranded RNA represented by SEQ. ID. NO: 50 (5'-UCU UUG GUU AUG UAG CUG UAU GA-3'); sense strand for siRNA duplex represented by sequence id. No. 51 (5'-UUA AGG CAC GCG GUG AAU GCC A-3'); antisense strand for siRNA duplex represented by SEQ. ID. NO: 52 (5'-GCA UUC ACC GCG UGC CUU AAU U-3').

The present inventors further investigated the relative affinity of pri-miRNA to DGCR8 by competition experiments. Internally labeled pri-miR-30a was

crosslinked to FLAG-DGCR8 in the presence of different amounts of unlabeled competitors such as siRNA duplex, 23 nt ssRNA, 80 bp dsRNA and pre-miR-30a hairpin. The nonlabeled pri-miR-30a competed with labeled pri-miR-30a, but the other RNA molecules did not compete efficiently in this reaction (Fig. 19). Pre-miR-30a had barely competed with pri-miR3-a, under these conditions suggesting that the main binding site for DGCR8 resides outside the upper stem and the terminal loop. This result also indicates that DGCR8 may dissociate from pre-miRNA upon processing. The long dsRNA has a relatively high affinity to DGCR8 and 23 nt ssRNA is also capable of competing with pri-miR-30a but inefficiently (Fig. 19). The above results indicate that DGCR8 may interact with pri-miRNAs by recognizing both ssRNA and dsRNA structures.

The present inventors further carried out crosslinking experiments using mutated pri-miRNAs prepared in Example 3 as cold competitors (Fig. 20). These mutants were disrupted either in their basal segments (single strand franking segment, 16- Δ BS) or in their terminal loop structures (16-TL7). The basal segment mutant (16- Δ BS) could not compete for the binding to DGCR8 with wild type RNA. The pri-miRNA containing a smaller terminal loop (16-TL7) was slightly impaired in this binding but was still able to compete with wild type RNA (Fig. 20). The above

results indicate that ssRNA segments of the pri-miRNAs are critical for DGCR8 binding.

In addition, the shorter-stem mutants (16-TL7/U-10 and 16-TL7/U-20) were tested to examine the requirements for the minimal stem length for DGCR8 binding (Fig. 20). The stem lengths of mutants 16-TL7/U-10 and 16-TL7/U-20 were predicted to be 31-33 bp and 21-23 bp, respectively. As the stem becomes shorter, the mutants competed gradually less efficiently than the longer mutant 16-TL7 (Fig. 20B).

Example 5: Preparation of artificial pri-miRNA

Strands A, B, C and D of artificial substrates for Microprocessor were synthesized (Samchully Pharmaceuticals, Korea) and then phosphorylated at the 5' end using T4 polynucleotide kinase (Takara, Japan) and [γ -³²P]ATP, for which the nucleotide sequences had been selected at random. The RNAs were heated in TE buffer at 95°C for 15 seconds and cooled down slowly.

The present inventors generated an artificial pri-miRNA molecule bearing no sequence homology to any known pri-miRNAs. When annealed, the two RNA strands of this molecule formed a simple structure of "ssRNA tail-3-helical turns-ssRNA tail" (Fig. 21). The two strands were then labeled at the 5' end in a given reaction to allow for easy

identification of the cleavage products. The efficiency of artificial pri-miRNA molecule was investigated. This artificial substrate was found to have been cleaved either at -11 bp from the left junction (cleavage 1) or at -11 bp from the right junction (cleavage 2) (Fig. 21). Then, strand A was replaced by strand B in order to convert the ssRNA tails in one side into an extended stem (Fig. 22, line 3). Cleavage on the left side was abolished, whereas cleavage on the right side was only slightly affected. When the ssRNA tail on the right side was also converted into a dsRNA stem, this simple dsRNA was not cleaved by Drosha (Fig. 22, line 4). The above results indicate that cleavage takes place at -11 bp from the dsRNA-ssRNA junction.

To examine whether DGCR8 binds to the ssRNA tails, the relative affinity of these RNAs for DGCR8 was determined by UV cross-linking competition experiments (Fig. 23). The duplexes containing ssRNA tails displayed the high affinity to DGCR8, whereas a simple duplex could not compete for this interaction effectively.

Example 6: Construction of a RNA interfering vector and the effect thereof

<6-1> Construction of a RNA interfering vector

The present inventors constructed AX backbone based on pri-miR-16-1 backbone. The vector was designed to be suitable for sub-cloning of a target mRNA with the insertion of a restriction enzyme without affecting the structure of pri-miR-16-1. From the investigation of sequences of the restriction enzyme site was confirmed that *Afl* II and *Xho* I restriction enzymes were selected (Fig. 25). The inventors further examined how the alteration of nucleotide sequence of AX backbone affected the expression of shRNA. At that time, pNKA57 that was used in Example <3-1> was used as a template to construct an expression vector for pri-miRNA by the same manner as described in Example 3. pcDNA3 was utilized as a backbone vector and the constructed vector was named "pcDNA3-pri-miRNA-1". Northern blotting (Sambrook J, et al., Molecular Cloning 2nd ED, Cold Spring Harbor Laboratory Press, 1989) was performed to investigate the level of the expression.

As a result, miR-16-1 was normally expressed from AX backbone, compared with the expression of wild type mi-miR16-1 (Fig. 26).

<6-2> The activity of ShLuci AX Flat

The present inventors introduced the nucleotide sequence of siRNA (5'-CGA AGU ACU CAG CGU AAG-3': SEQ. ID. NO: 53) into the RNA interfering vector prepared in Example

<6-1>, resulting in "shLuci_AX_Flat" (Fig. 27). Then, the inventors examined whether the produced shRNA could inhibit the activity of luciferase. HEK293T cells were transfected with the shLuci_AX_Flat, followed by examining the activity of luciferase by using Luciferase assay system (Promega, USA) according to the manufacturer's instruction.

As a result, shRNA of the present invention efficiently inhibited the activity of luciferase (Fig. 28).

<6-3> Alteration of the structure of shLuci AX Flat and measurement of the activity of the same

The present inventors altered the structure of shLuci_AX_Flat by the same manner as described in Example 3. The alteration was carried out as follows: alteration of the terminal loop (shLuci_AX_Flat_TL: Fig. 29); alteration of the internal loop (shLuci_AX_Flat_IL: Fig. 30); alteration of the internal loop and bulge (shLuci_AX_Flat_IL/B: Fig. 31); and miR30-like alteration (shLuci_AX_Flat_30like: Fig. 32). Then, the activity of luciferase of each variant was measured by the same manner as described in Example <6-2>.

As a result, shLUCI_AX_Flat variants reduced the activity of luciferase efficiently (Fig. 33).

【Industrial Applicability】

According to the preparing method of the recombinant primary-microRNA molecule for RNA interference of the present invention, the recombinant primary-microRNA molecule can be successfully designed to induce its target gene silencing efficiently.

【Sequence List Text】

SEQ. ID. NO: 1 - NO: 49 are oligomers used for the generation of mutants of the invention.

SEQ. ID. NO: 50 is a single stranded nucleotide sequence of siRNA.

SEQ. ID. NO: 51 and NO: 52 are nucleotide sequences of siRNA duplex.

SEQ. ID. NO: 53 is siRNA sequence of luciferase.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

【CLAIMS】**【Claim 1】**

A recombinant pri-miRNA (primary-microRNA) molecule
containing successively a single strand tail; an 8 - 14 bp
5 long lower stem; an upper stem containing its target
sequence specific sequence; and a terminal loop.

【Claim 2】

The recombinant pri-miRNA molecule according to claim
10 1, wherein the single strand tail is composed of the 5' end
strand and/or the 3' end strand.

【Claim 3】

The recombinant pri-miRNA molecule according to claim
15 2, wherein the 5' end strand does not correspond to the 3'
end strand.

【Claim 4】

The recombinant pri-miRNA molecule according to claim
20 1, wherein the length of the single strand tail is more
than 5 nt.

【Claim 5】

The recombinant pri-miRNA molecule according to claim 4, wherein the length of the single strand tail is 8 - 14 nt.

5 **【Claim 6】**

The recombinant pri-miRNA molecule according to claim 1, wherein the length of the lower stem is 11 bp.

【Claim 7】

10 The recombinant pri-miRNA molecule according to claim 1, wherein the length of the upper stem is 10 - 30 bp.

【Claim 8】

15 The recombinant pri-miRNA molecule according to claim 7, wherein the length of the upper stem is 17 - 24 bp.

【Claim 9】

20 The recombinant pri-miRNA molecule according to claim 1, wherein the upper stem characteristically contains a mismatch containing 1 - 3 internal loops or a bulge.

【Claim 10】

25 The recombinant pri-miRNA molecule according to claim 9, wherein the upper stem characteristically contains a mismatch containing 1 - 2 internal loops or a bulge.

【Claim 11】

The recombinant pri-miRNA molecule according to claim 1, wherein the upper stem contains a mismatch containing 1 - 3 internal loops or a bulge in its target gene specific sequence region.

【Claim 12】

The recombinant pri-miRNA molecule according to claim 11, wherein the mismatch resides at the position of 9th - 15th nt of the upper stem and the size of the mismatch is 1 - 3 nt and the numbers of the mismatch are 1 - 4.

【Claim 13】

The recombinant pri-miRNA molecule according to claim 1, wherein the lower stem contains a mismatch containing 1 - 3 internal loops or a bulge.

【Claim 14】

The recombinant pri-miRNA molecule according to claim 13, wherein the lower stem characteristically contains a mismatch containing an internal loops or a bulge.

【Claim 15】

The recombinant pri-miRNA molecule according to claim 1, wherein the terminal loop links the double strand of the upper stem.

5 **【Claim 16】**

The recombinant pri-miRNA molecule according to claim 1, wherein the length of the terminal loop is 1 - 20 nt.

【Claim 17】

10 The recombinant pri-miRNA molecule according to claim 16, wherein the length of the terminal loop is 4 - 18 nt.

【Claim 18】

15 The recombinant pri-miRNA molecule according to claim 1, wherein the junction of the upper stem and the lower stem is cleaved by Drosha-DGCR8 complex.

【Claim 19】

20 A recombinant pri-miRNA molecule containing successively a single strand tail; an 8 - 14 long lower stem; an upper stem bearing its target gene specific sequence; a single strand head.

【Claim 20】

The recombinant pri-miRNA molecule according to claim 19, wherein the single strand tail and the single strand head are composed of the 5' end strand and/or the 3' end strand.

5

【Claim 21】

The recombinant priming molecule according to claim 20, wherein the 5' end strand and the 3' end strand of the single strand tail and the single strand head do not correspond each other.

10

【Claim 22】

The recombinant pri-miRNA molecule according to claim 19, wherein the lengths of the single strand tail and the single strand head are more than 5 nt.

15

【Claim 23】

The recombinant pri-miRNA molecule according to claim 22, wherein the lengths of the single strand tail and the single strand head are 8 - 14 nt.

20

【Claim 24】

The recombinant pri-miRNA molecule according to claim 19, wherein the length of the lower stem is 11 bp.

25

【Claim 25】

The recombinant pri-miRNA molecule according to claim 19, wherein the length of the upper stem is 10 - 30 bp.

5 **【Claim 26】**

The recombinant pri-miRNA molecule according to claim 25, wherein the length of the upper stem is 17 - 24 bp.

【Claim 27】

10 The recombinant pri-miRNA molecule according to claim 19, wherein the upper stem characteristically contains a mismatch containing 1 - 3 internal loops or a bulge.

【Claim 28】

15 The recombinant pri-miRNA molecule according to claim 27, wherein the upper stem characteristically contains a mismatch containing 1 - 2 internal loops or a bulge.

【Claim 29】

20 The recombinant pri-miRNA molecule according to claim 19, wherein the upper stem characteristically contains a mismatch containing an internal loop and a bulge in its target gene specific sequence region.

25 **【Claim 30】**

The recombinant pri-miRNA molecule according to claim 29, wherein the mismatch resides at the position of 9th - 15th nt of the upper stem and the size of the mismatch is 1 - 3 nt and the numbers of the mismatch are 1 - 4.

5

【Claim 31】

The recombinant pri-miRNA according to claim 19, wherein the lower stem characteristically contains a mismatch containing 1 - 2 internal loops or a bulge.

10

【Claim 32】

The recombinant pri-miRNA molecule according to claim 31, wherein the lower stem contains a mismatch containing an internal loop or a bulge.

15

【Claim 33】

The recombinant pri-miRNA molecule according to claim 19, wherein the junction of the upper stem and the lower stem is cleaved by Drosha-DGCR8 complex.

20

【Claim 34】

A polynucleotide molecule encoding the nucleotide sequence of the recombinant pri-miRNA molecule of claim 1.

25

【Claim 35】

The polynucleotide molecule according to claim 34, which is characteristically composed of the sequence of the 5' end; the sense sequence of a lower stem; the sense sequence of an upper stem containing its target gene specific sequence; the sequence of a terminal loop; the antisense sequence of an upper stem containing its target gene specific sequence; the antisense sequence of a lower stem; and the sequence of the 3' end in that order.

10 **【Claim 36】**

A polynucleotide molecule encoding the nucleotide sequence of the pri-miRNA molecule of claim 19.

15 **【Claim 37】**

15 The polynucleotide molecule according to claim 36, which is characteristically composed of the sequence of the 5' end; the sense sequence of a lower stem; the sense sequence of an upper stem containing its target gene specific sequence; the upper strand encoding the sequence of the 3' end and the sequence of the 3' end; the antisense sequence of a lower stem; the antisense sequence of an upper stem containing its target gene specific sequence; the lower strand encoding the sequence of the 5' end in that order.

【Claim 38】

A recombinant expression vector harboring the polynucleotide of claim 34.

5 **【Claim 39】**

The recombinant expression vector according to claim 38, wherein the expression vector is selected from a group consisting of plasmid, lentivirus vector, retrovirus vector and adenovirus vector.

10

【Claim 40】

The recombinant expression vector according to claim 38, wherein the vector is generated using a RNA polymerase II promoter selected from a group consisting of
15 cytomegalovirus immediate early promoter, SV40 promoter, human immunodeficiency virus LTR and c-myc promoter.

【Claim 41】

A recombinant expression vector harboring the
20 polynucleotide of claim 36.

【Claim 42】

The recombinant expression vector according to claim 41, wherein the expression vector is selected from a group

consisting of plasmid, lentivirus vector, retrovirus vector and adenovirus vector.

【Claim 43】

5 The recombinant expression vector according to claim 41, wherein the vector is generated using a RNA polymerase II promoter selected from a group consisting of cytomegalovirus immediate early promoter, SV40 promoter, human immunodeficiency virus LTR and c-myc promoter.

10

【Claim 44】

A therapeutic agent containing the recombinant pri-miRNA molecule of claim 19 and/or the recombinant expression vector of claim 38 and/or claim 41.

15

【Claim 45】

The therapeutic agent according to claim 44, wherein the applicable disease is selected from a group consisting of cancer, viral infection, genetic disease, metabolic disease, immune disease, neurodegenerative disease and ophthalmology disease.

20

【Claim 46】

A preparing method for the recombinant pri-miRNA molecule of claim 1 comprising the following steps:

25

1) Selecting mRNAs of target genes;

2) Determining the pri-microRNA to be composed of 5' end sequence; sense sequence of a lower stem; sense sequence of a upper stem containing a target gene specific sequence; a terminal loop sequence; antisense sequence of a upper stem containing a target gene sequence; antisense sequence of a lower stem; and 3' end sequence successively;

3) Synthesizing the recombinant pri-miRNA molecule of step 2); and

4) Annealing the pri-miRNA molecule synthesized in step 3) to predict the secondary structure of the molecule.

【Claim 47】

A preparing method for the recombinant pri-miRNA molecule of claim 19 comprising the following steps:

1) Selecting mRNAs of target genes;

2) Determining the sequences of the upper strand RNA molecule harboring successively the 5' end sequence; the sense sequence of a lower stem; the sense sequence of an upper stem containing a target gene specific sequence; and the 3' end sequence of a upper stem and the lower strand RNA molecule harboring serially the 3' end sequence, the antisense sequence of a lower stem, the antisense sequence of a upper stem harboring a target gene specific sequence and the 5' end sequence of a lower stem;

3) Synthesizing the upper strand RNA and the lower strand RNA determined in the above step 2); and

4) Annealing the synthesized upper strand and the lower strand RNAs to predict the secondary structure.

5

【Claim 48】

A preparing method for the recombinant expression vector of claim 38 comprising the following steps:

1) Selecting mRNAs of target genes;

10 2) Determining the pri-miRNA to be composed of the 5' end sequence; the sense sequence of a lower stem; the sense sequence of a upper stem containing a target gene specific sequence; a terminal loop sequence; the antisense sequence of a upper stem containing a target gene sequence; the
15 antisense sequence of a lower stem; and the 3' end sequence successively;

3) Synthesizing a single stranded DNA strand corresponding to the recombinant pri-mRNA of step 2) and an antisense single stranded DNA strand thereof;

20 4) Annealing the single stranded DNA strands synthesized in step 3) to prepare a double stranded DNA; and

5) Linking the double stranded DNA of step 4) operably to the promoter of an expression vector.

1) selecting mRNAs of target genes; 2) determining the sequences of the recombinant pri-miRNA serially which are the sequence of the 5' end; sense sequence of the lower stem; sense sequence of the upper stem bearing its target gene specific sequence; sequence of the terminal loop; antisense sequence of the upper stem bearing its target gene specific sequence; antisense sequence of the lower stem; and the sequence of the 3' end; 3) synthesizing the single stranded DNA and its antisense single stranded DNA corresponding to those of the recombinant pri-miRNA molecule determined in the above step 2); 4) generating double-stranded DNA by annealing the synthesized single stranded DNAs to be paired; and 5) linking the double stranded DNA operably to the promoter of an expression vector.

【Claim 49】

The preparing method for the recombinant expression vector of claim 38 according to claim 48, wherein the expression vector of step 5) is selected from a group consisting of plasmid, lentivirus vector, retrovirus vector and adenovirus vector.

【Claim 50】

A preparing method for the recombinant expression vector of claim 41 comprising the following steps:

1) Selecting mRNAs of target genes;

2) Determining the sequences of the upper strand RNA molecule harboring successively the 5' end sequence; the sense sequence of a lower stem; the sense sequence of a upper stem containing a target gene specific sequence; and the 3' end sequence of a upper stem and the lower strand RNA molecule harboring serially the 3' end sequence, the antisense sequence of a lower stem, the antisense sequence of a upper stem harboring a target gene specific sequence and the 5' end sequence of a lower stem;

3) Synthesizing a single stranded DNA and its antisense single stranded DNA corresponding to the nucleotide sequences of the upper strand RNA and the lower strand RNA determined in step 2);

4) Annealing the single stranded DNA strands synthesized in step 3) to form a double stranded DNA molecule by base-pairing; and

5) Linking the double stranded DNA of step 4) operably to the promoter of an expression vector.

【Claim 51】

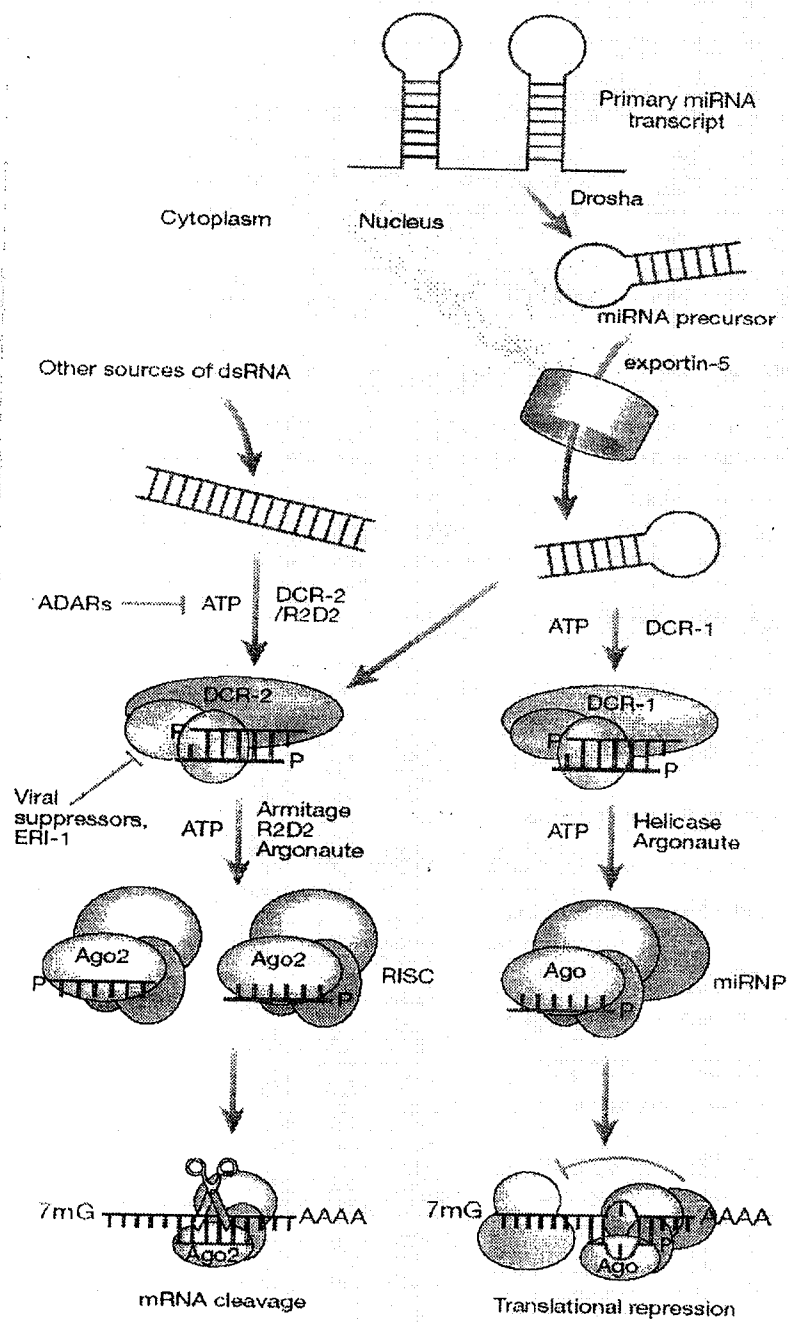
The preparing method for the recombinant expression vector of claim 41 according to claim 50, wherein the

expression vector of step 5) is selected from a group consisting of plasmid, lentivirus vector, retrovirus vector and adenovirus vector.

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Figures

Fig. 1



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Fig. 2

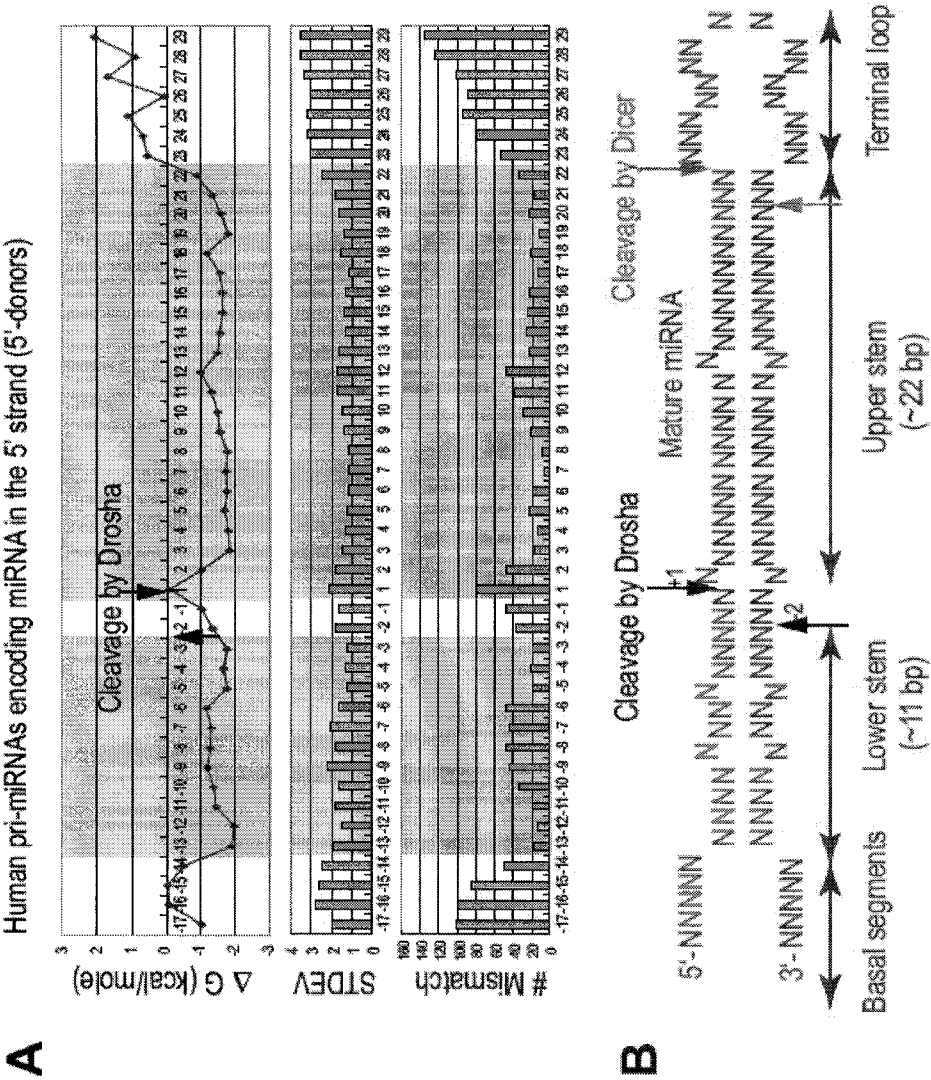


Fig. 3

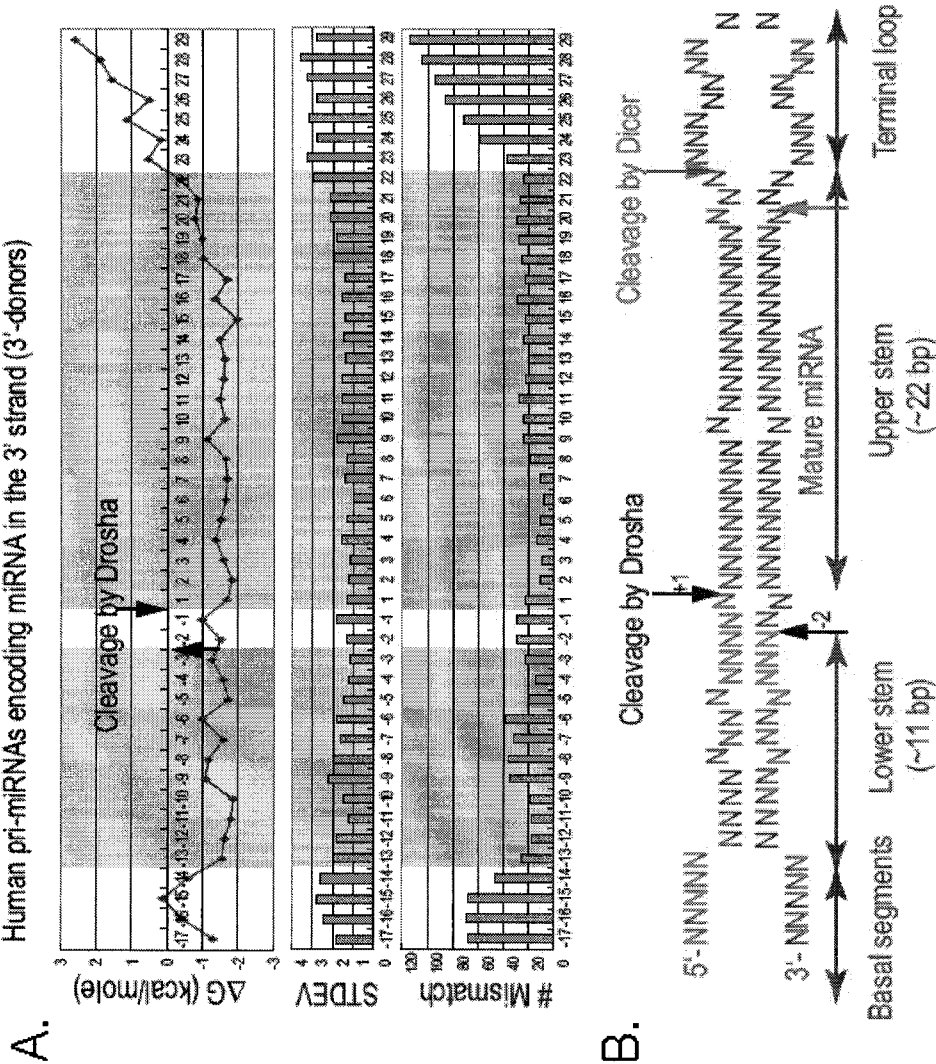
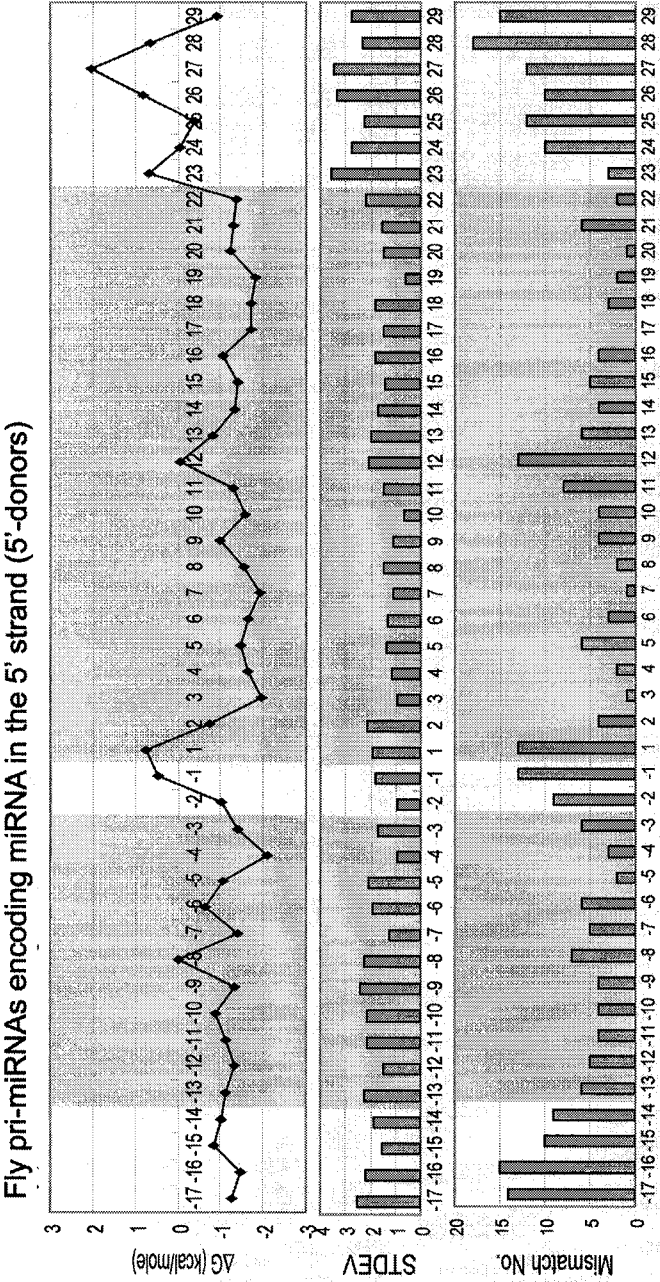
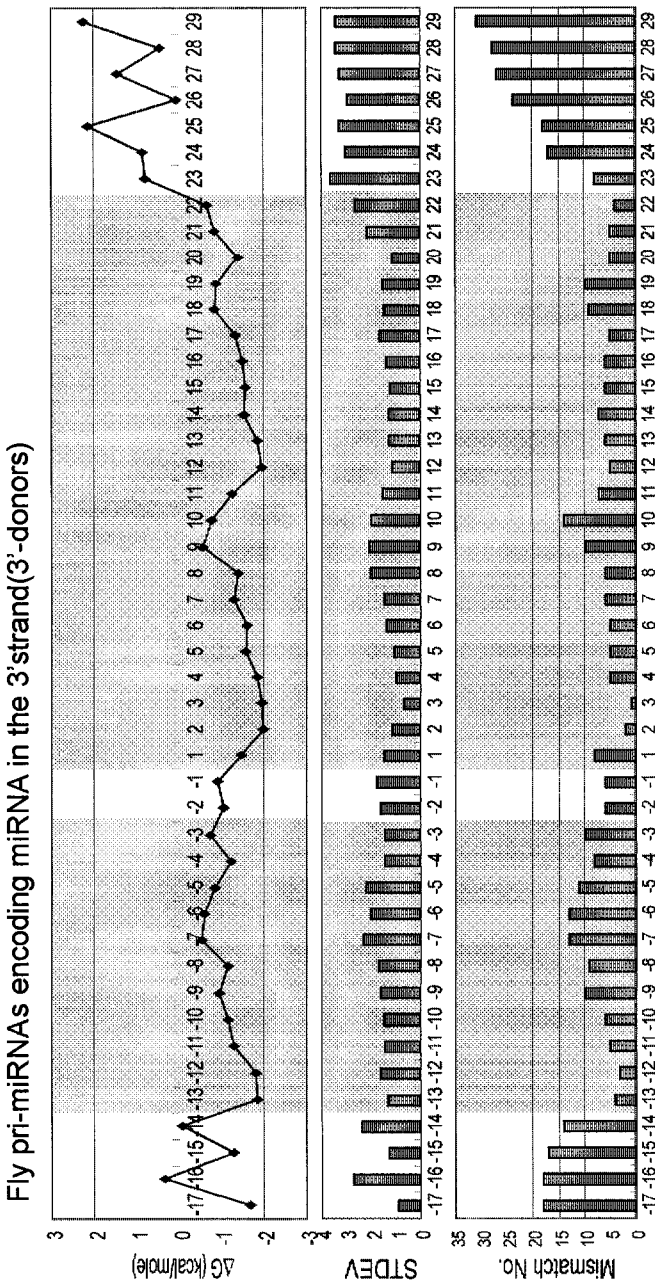


Fig. 4



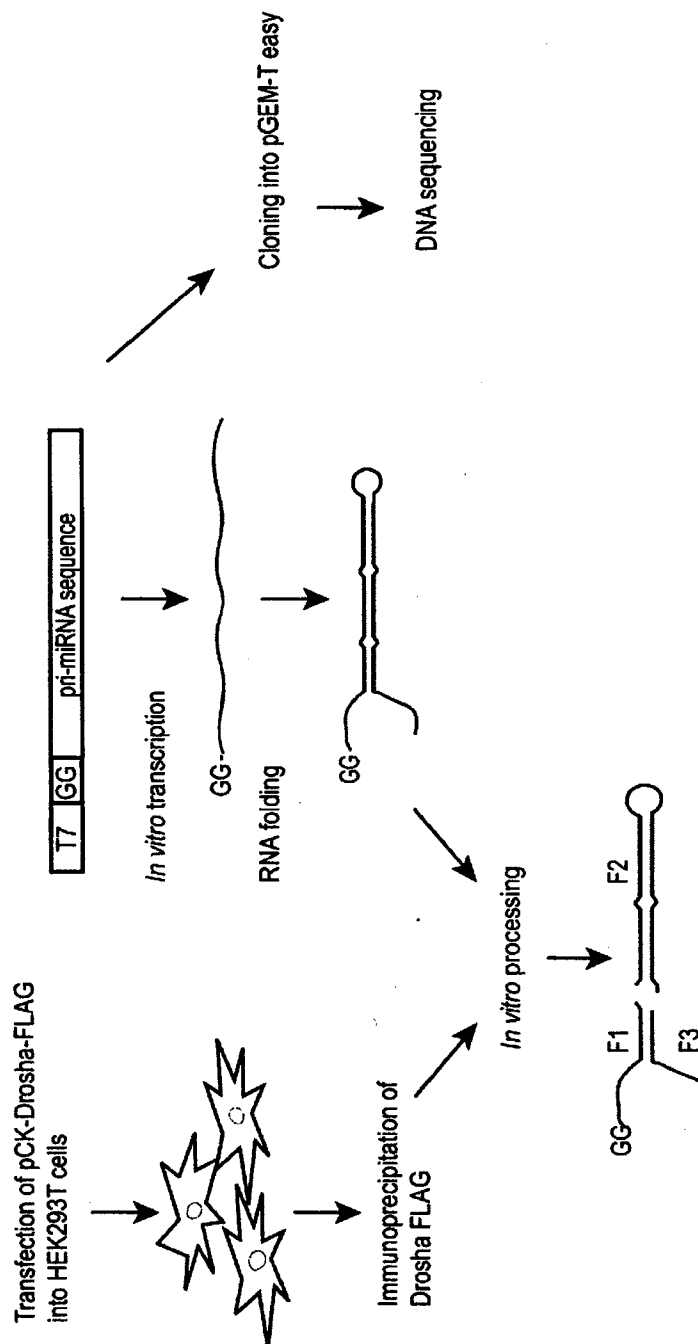
5/32

Fig. 5



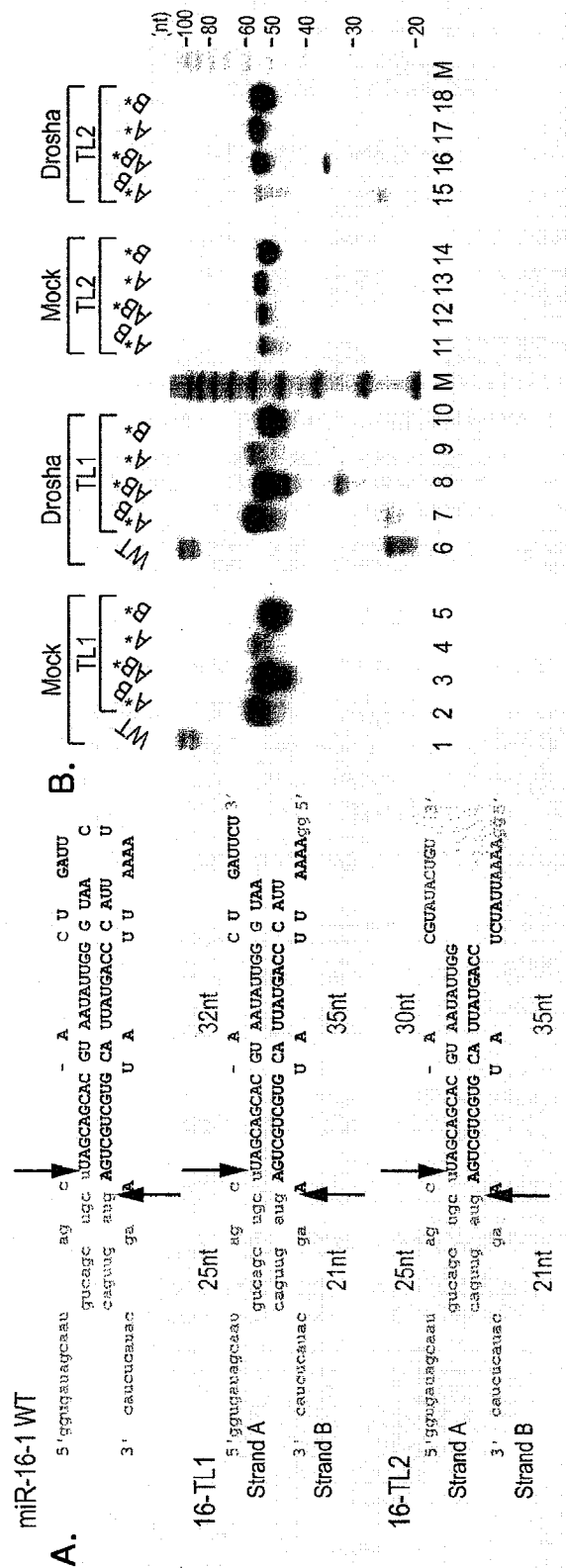
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Fig. 6



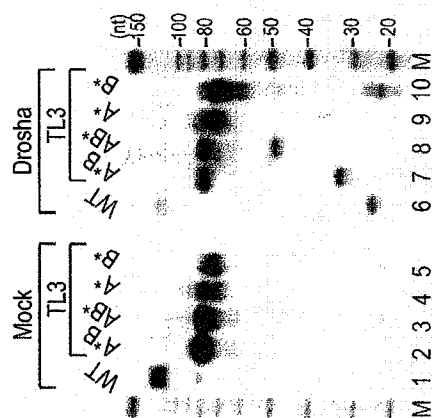
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Fig. 7

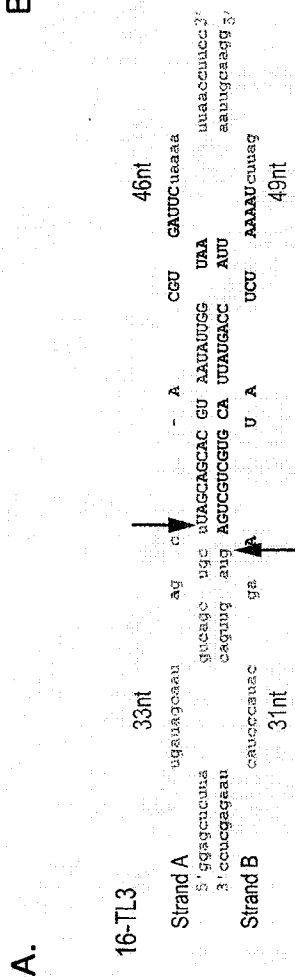


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Fig. 8

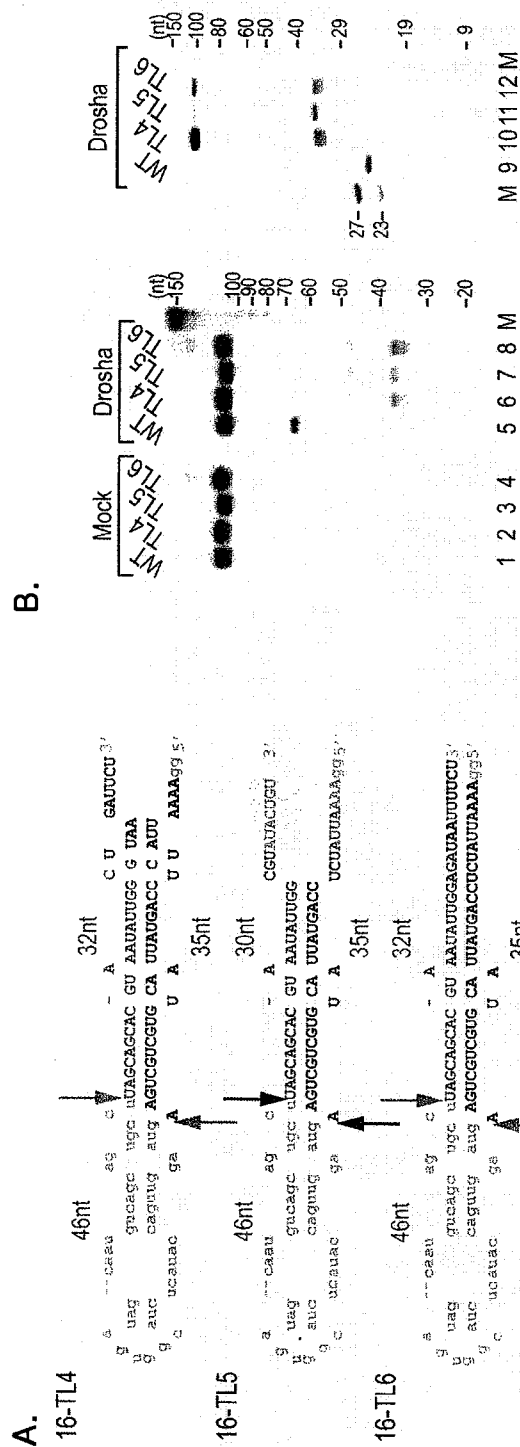


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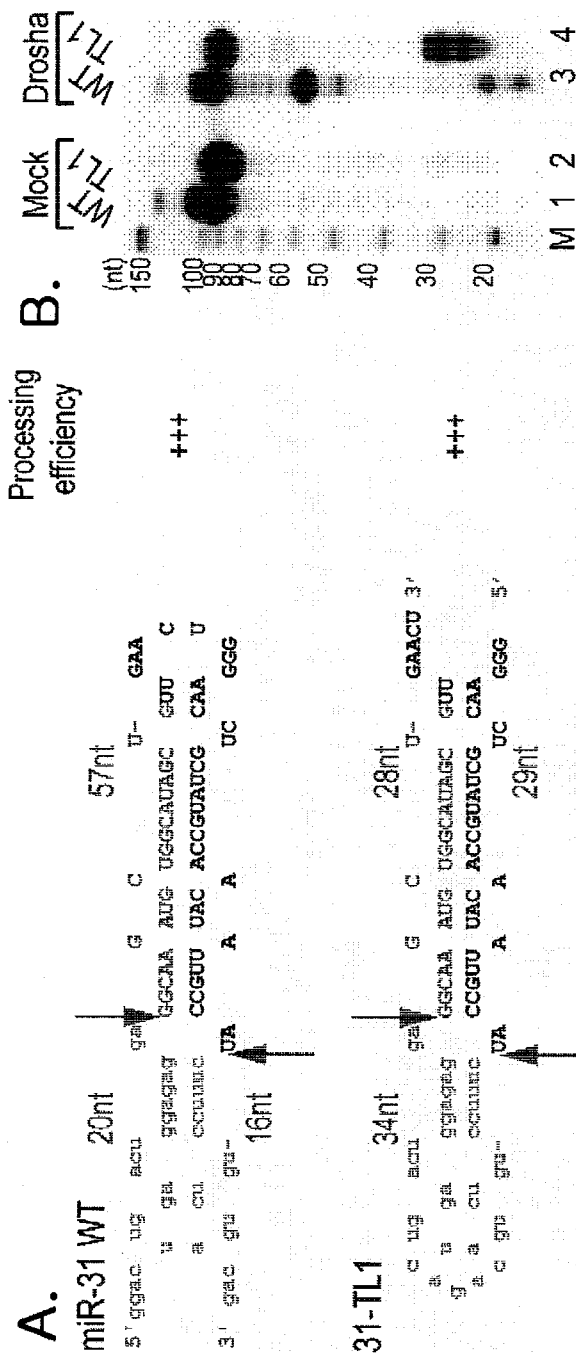
9/32

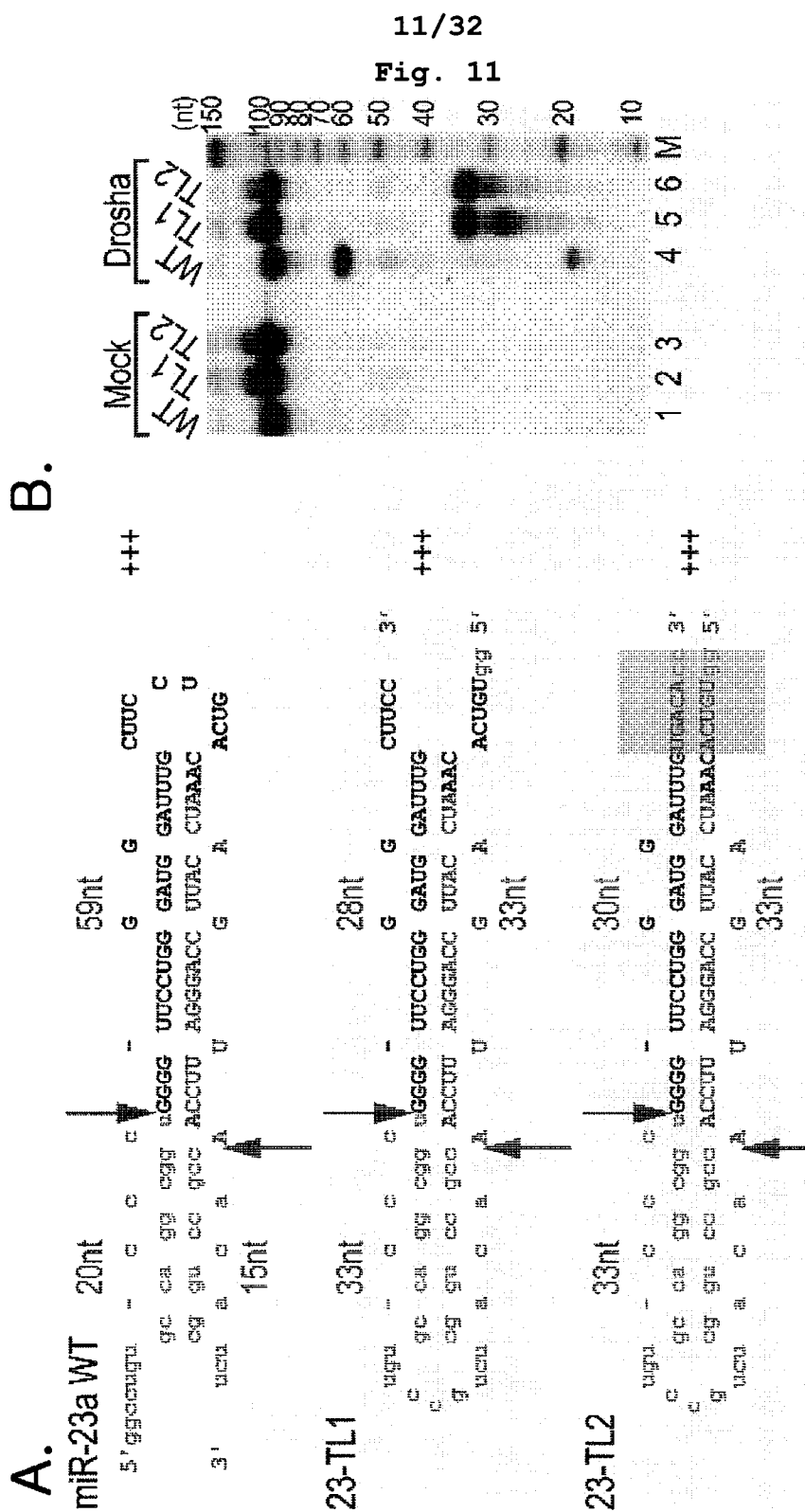
Fig. 9



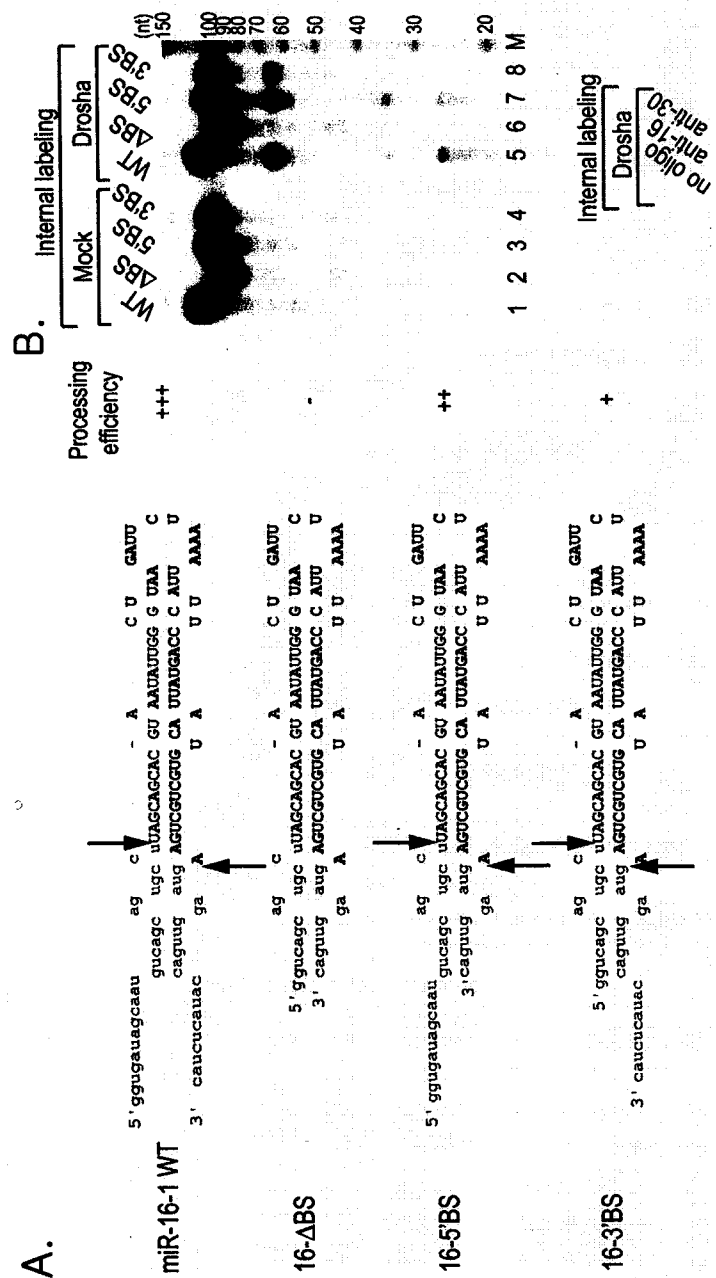
10/32

Fig. 10

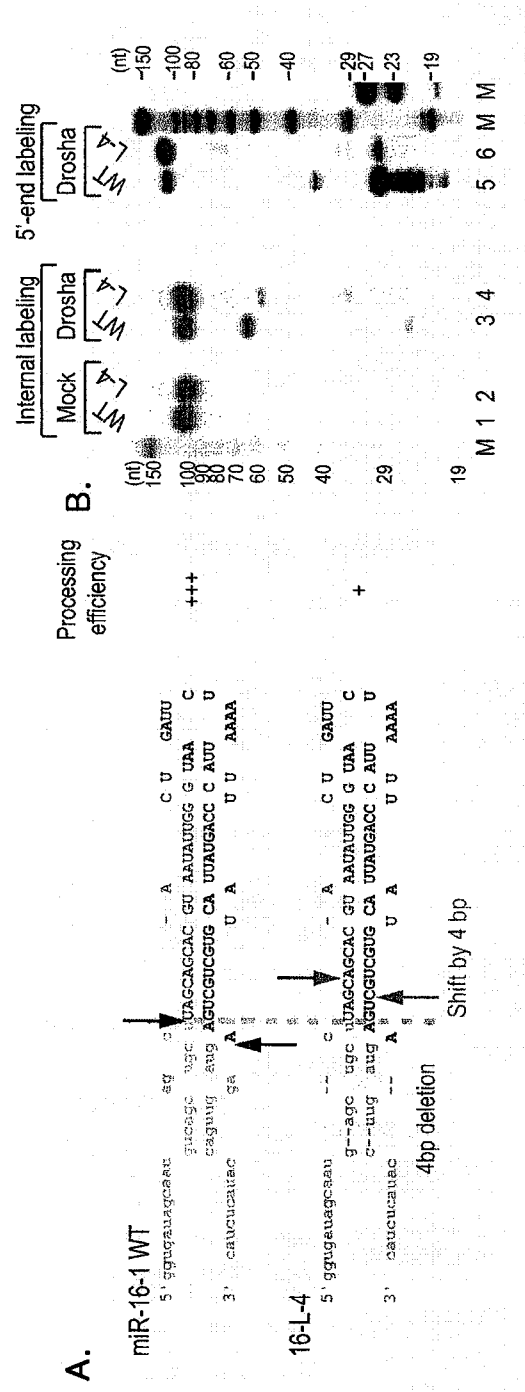




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Fig. 12

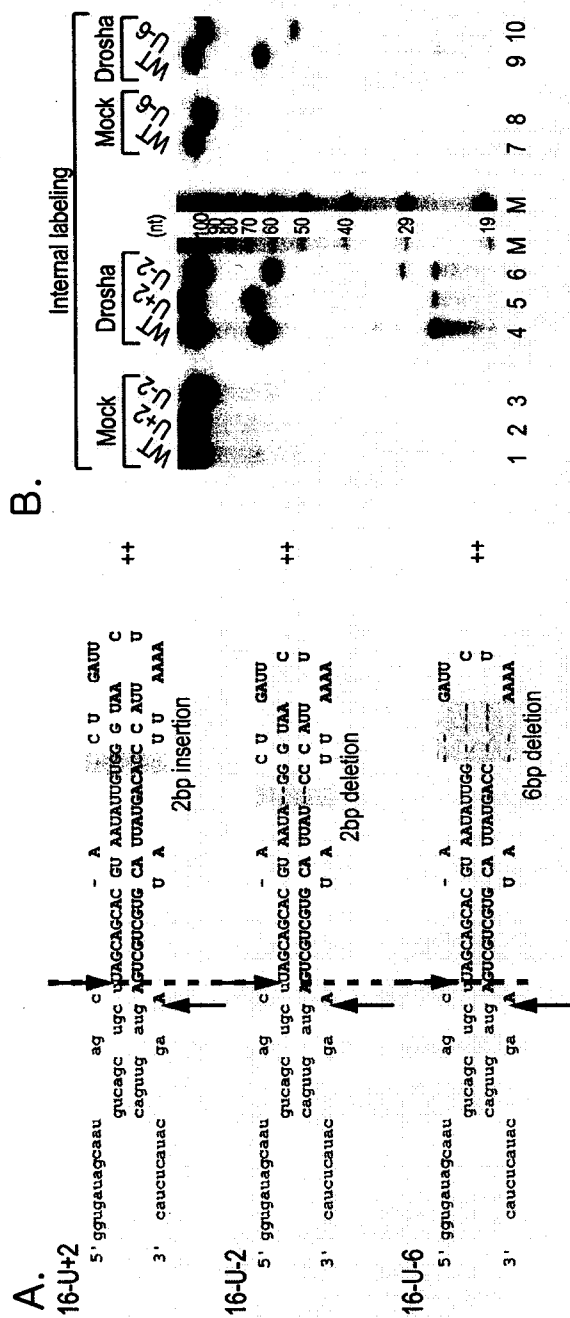


14/32
Fig. 14

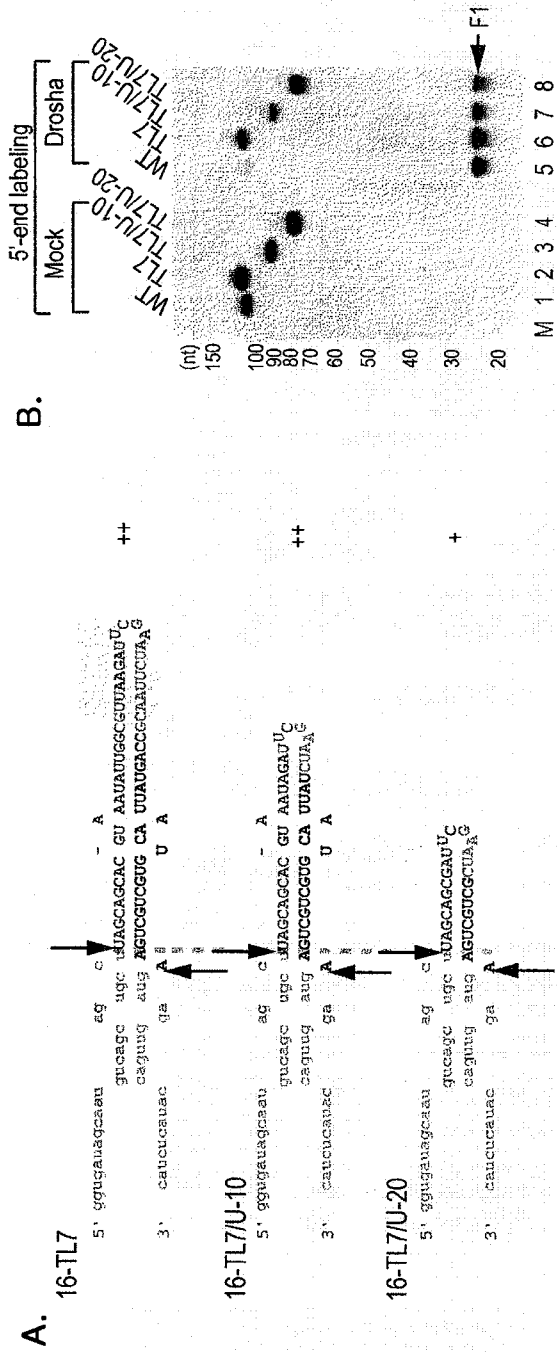


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Fig. 15



16/32
Fig. 16



17/32

Fig. 17

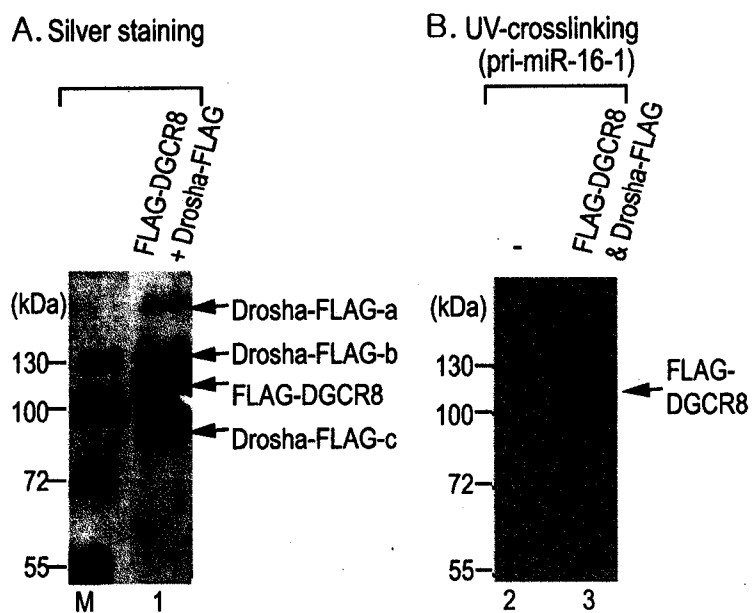
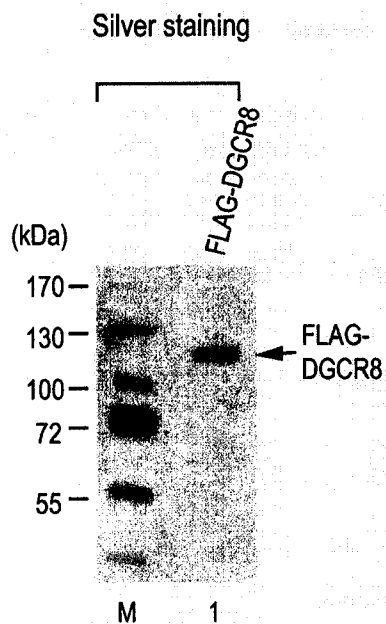


Fig. 18



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Fig. 19

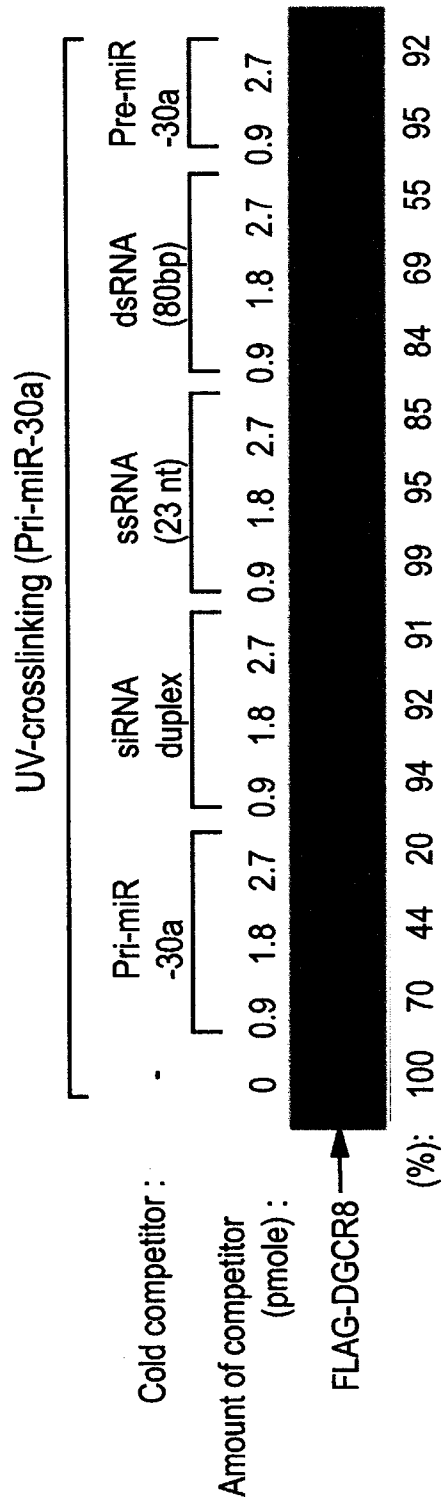


Fig. 20

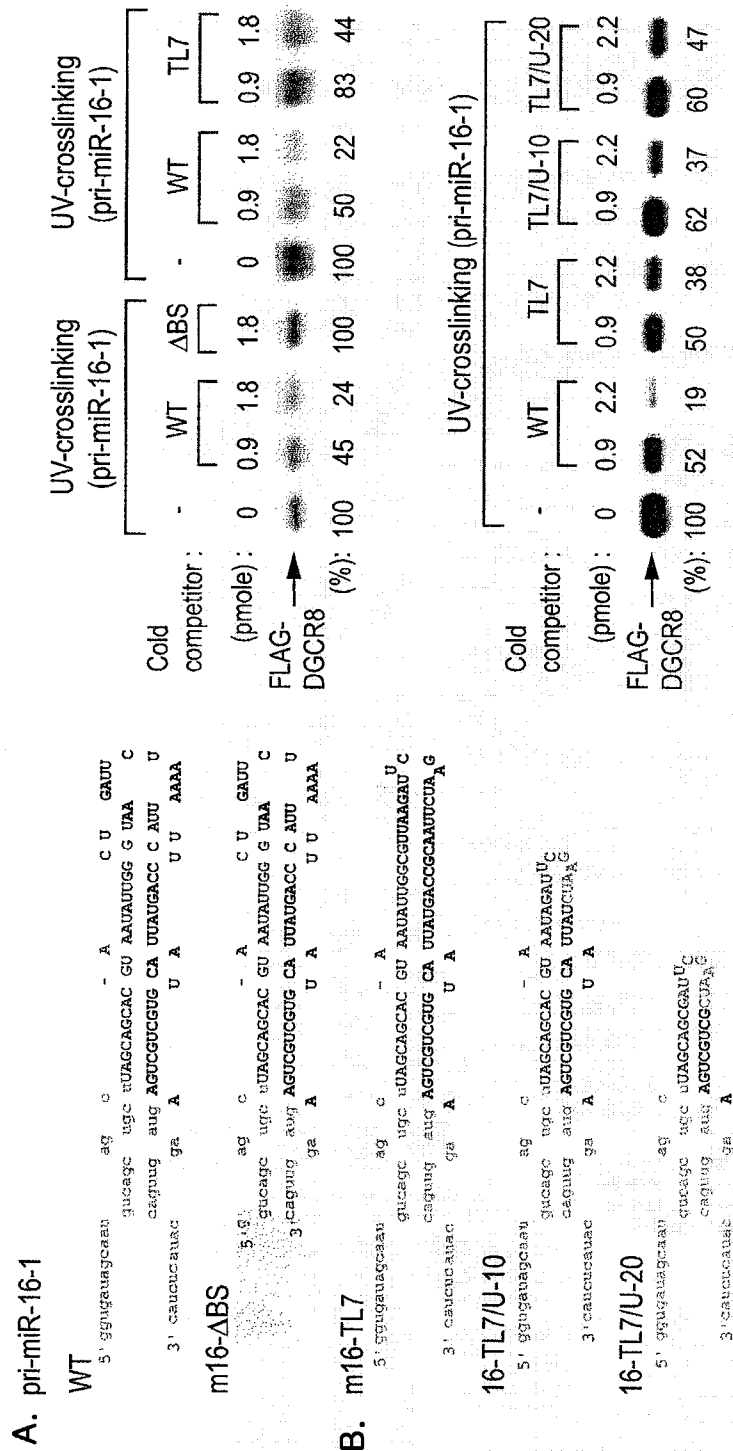
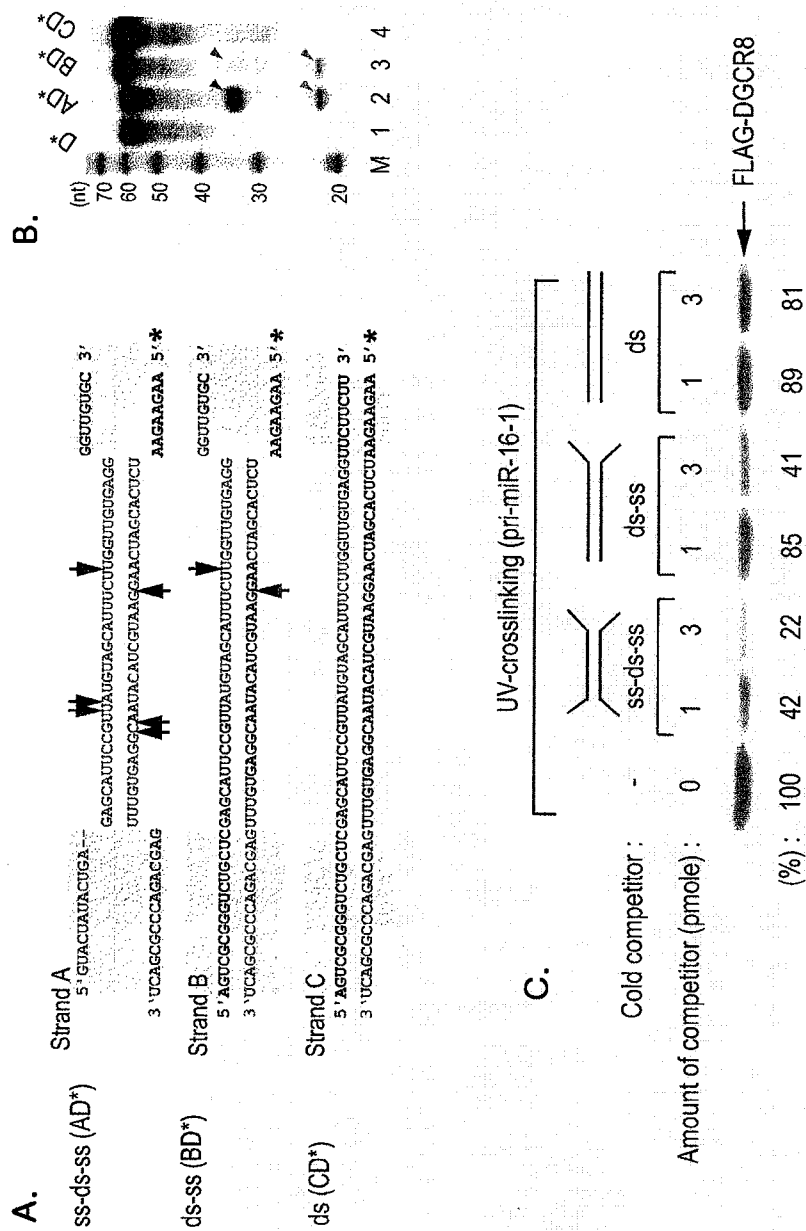
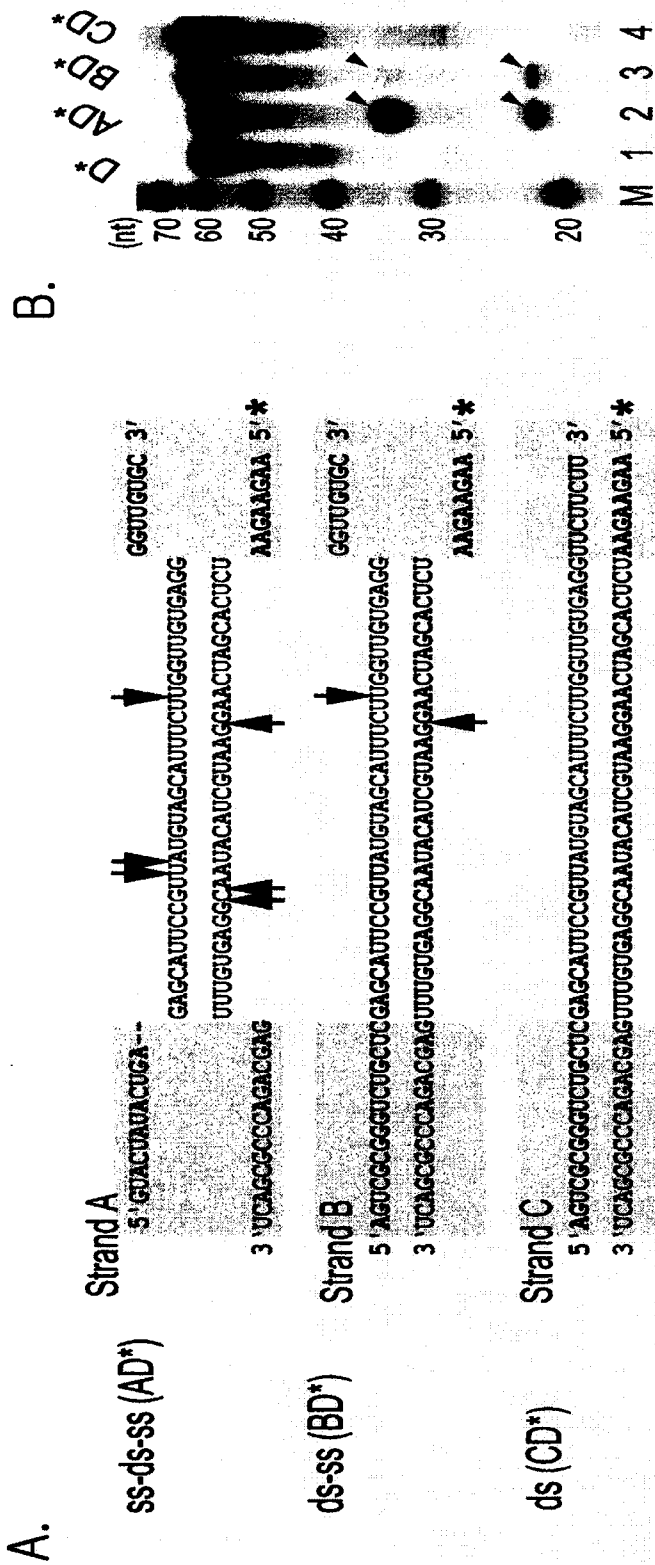


Fig. 21

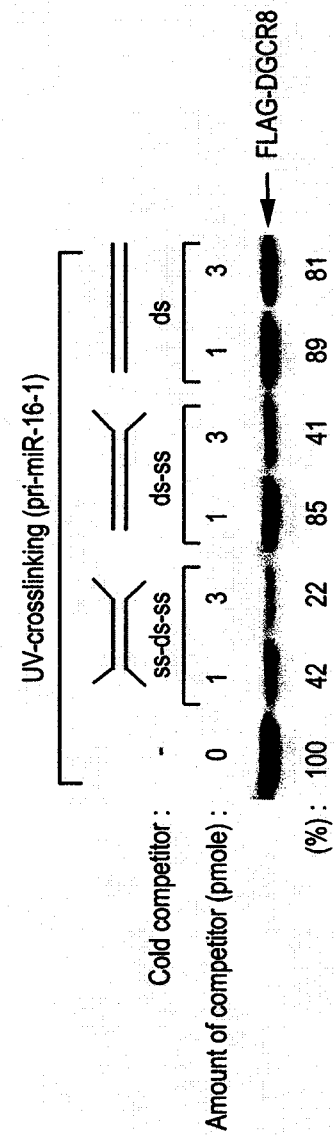


21/32
Fig. 22



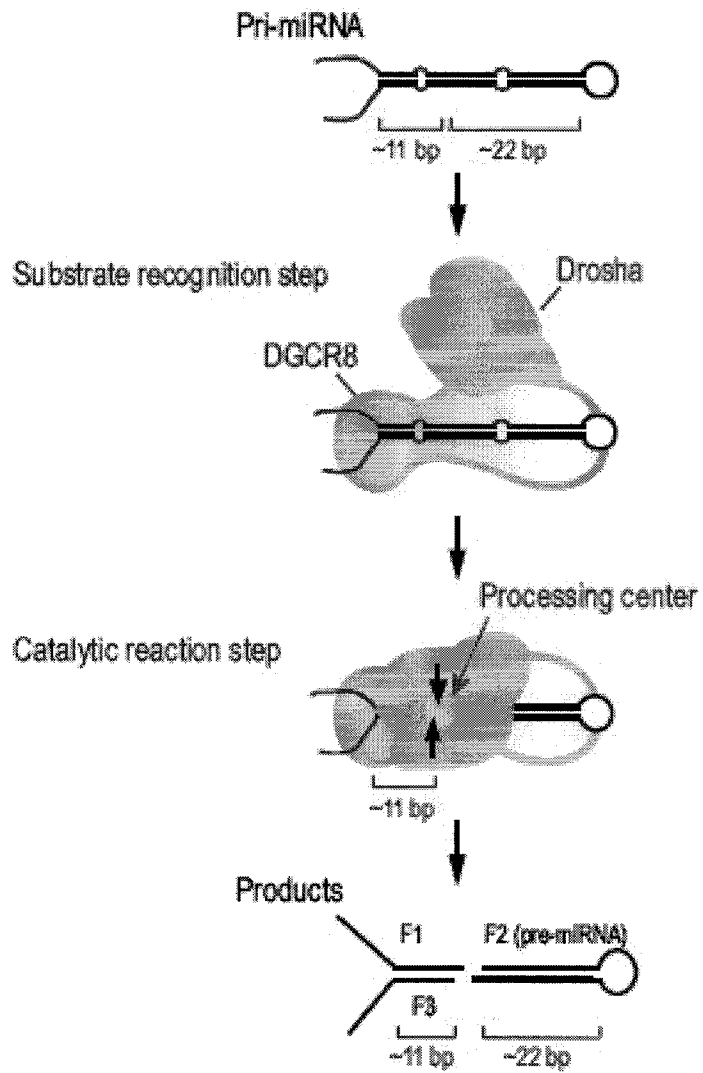
22/32

Fig. 23



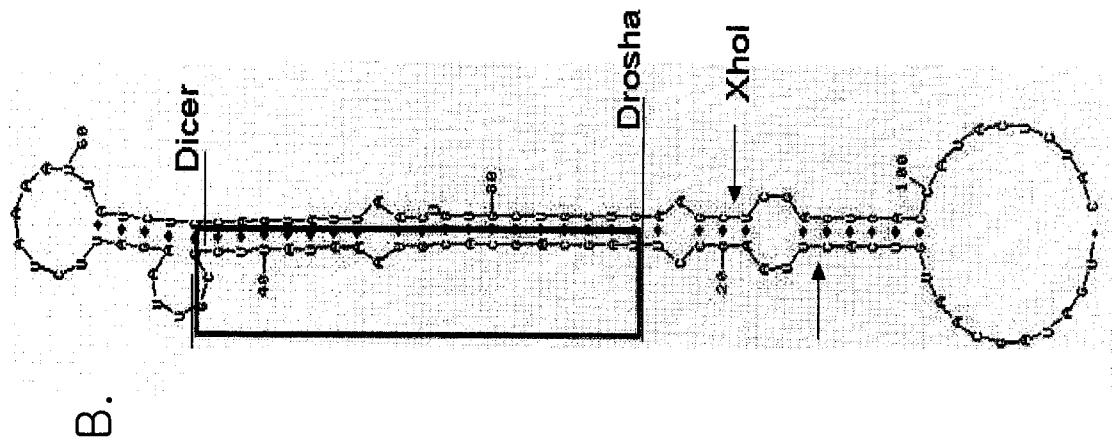
23/32

Fig. 24



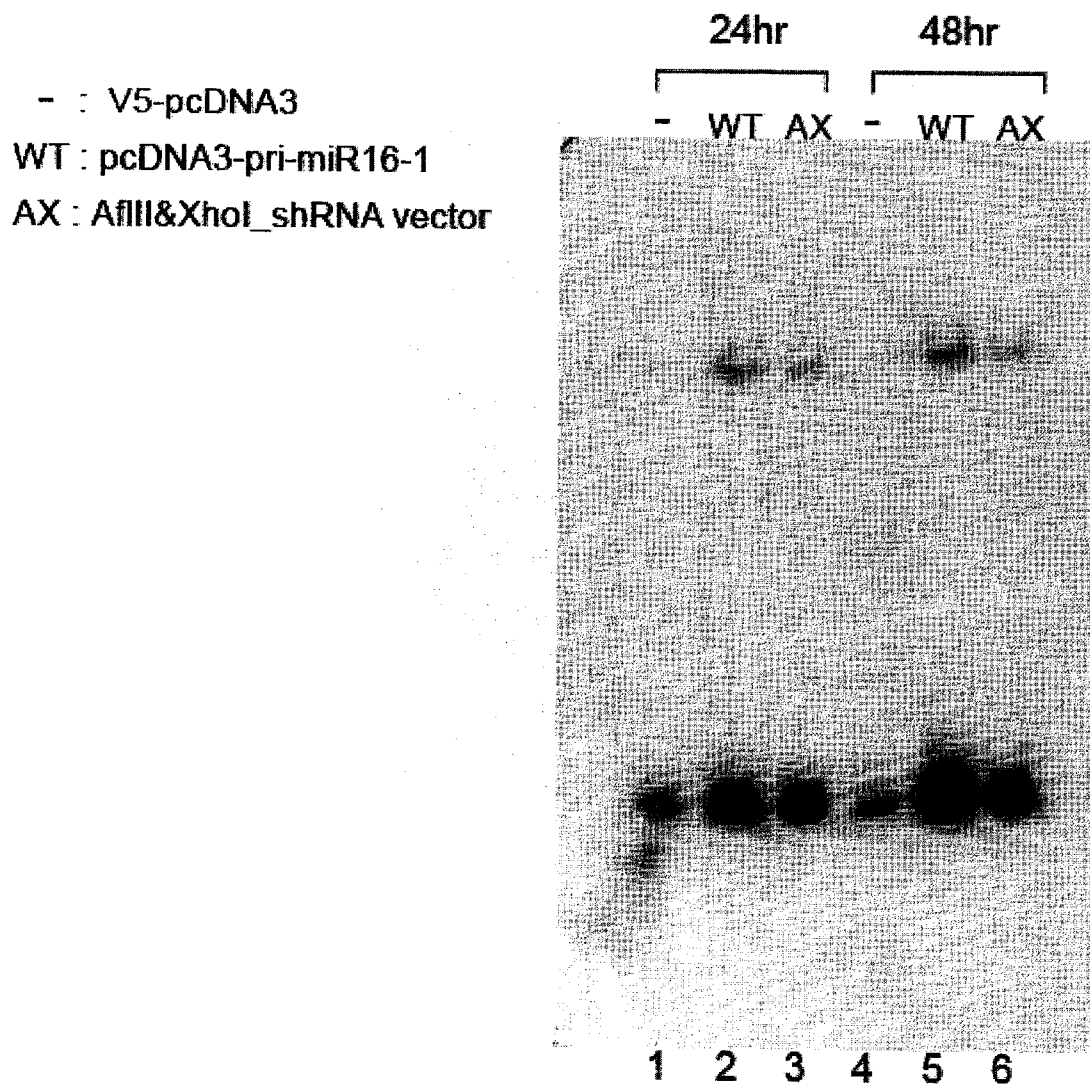
24/32

Fig. 25



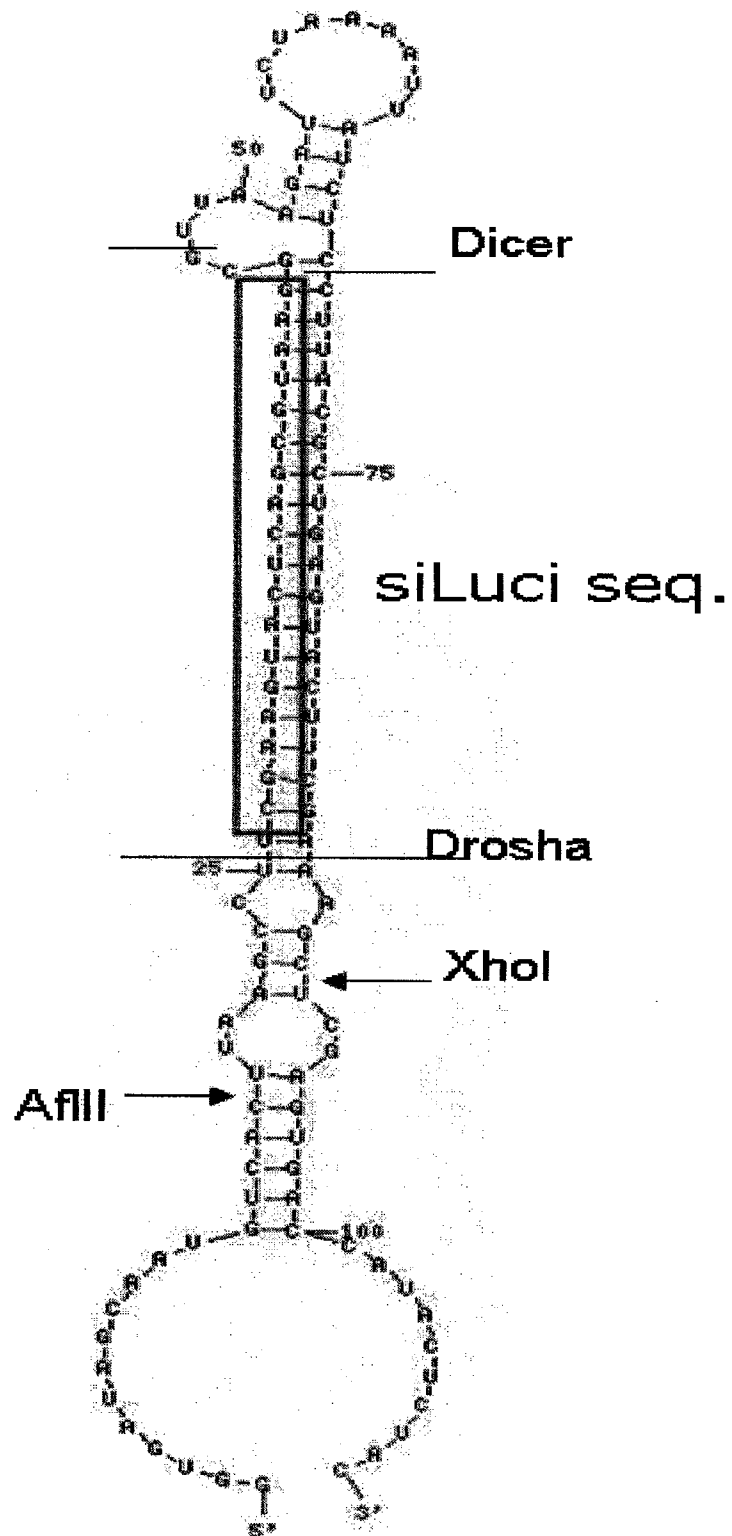
25/32

Fig. 26

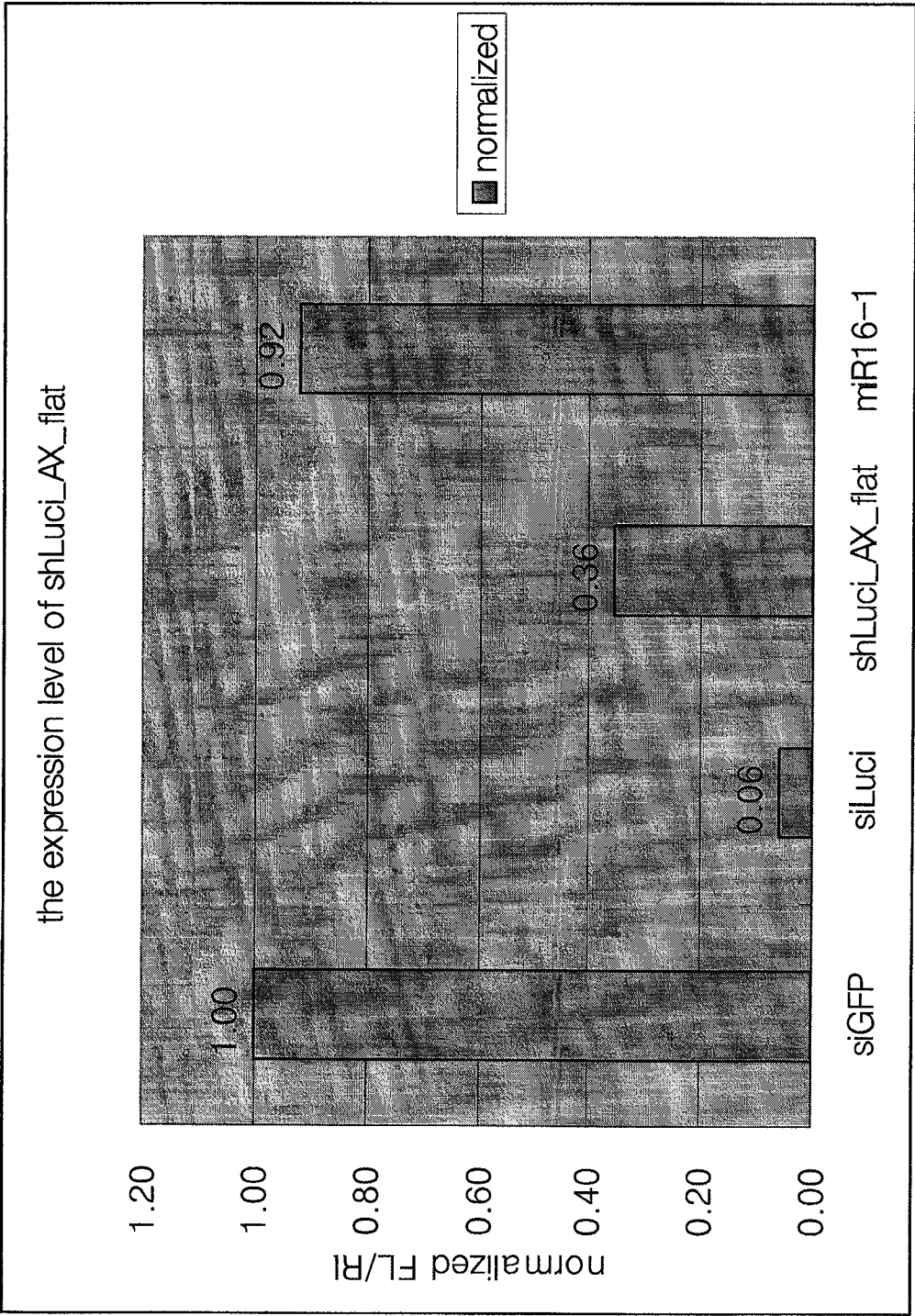


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Fig. 27

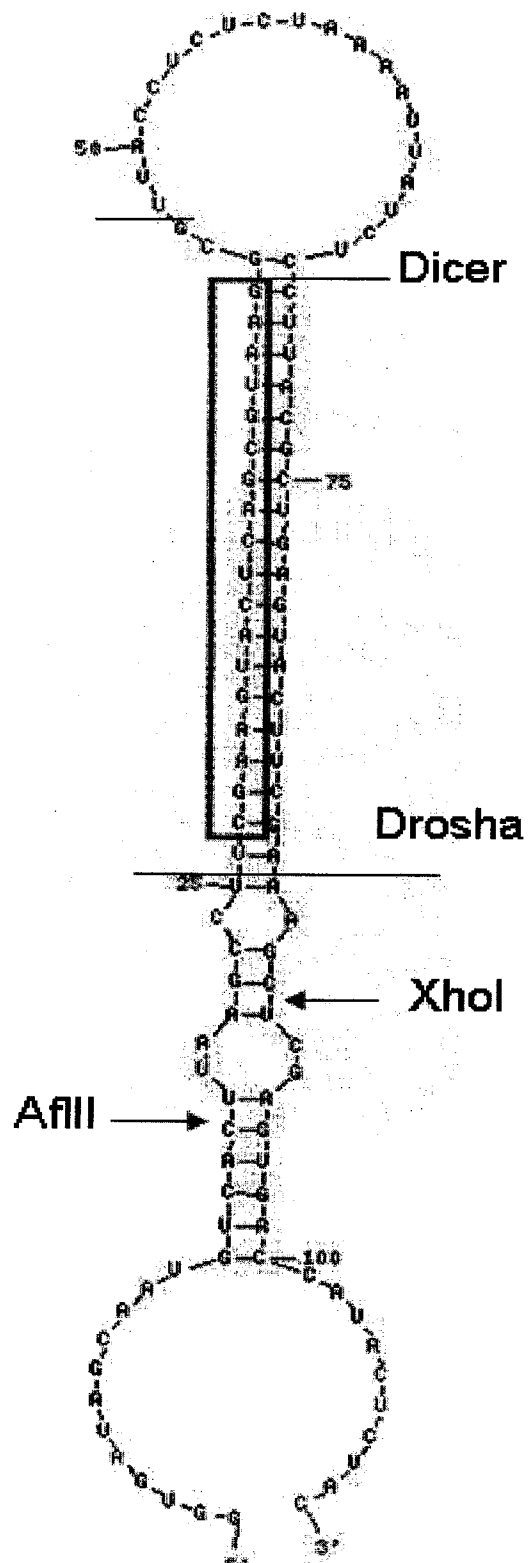


27/32
Fig. 28



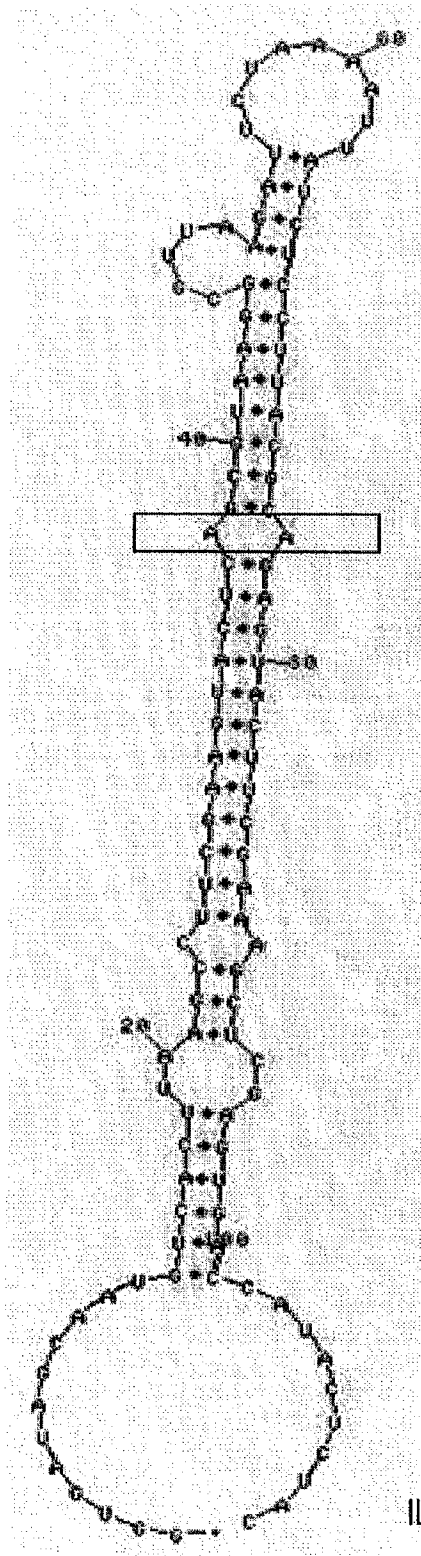
28/32

Fig. 29



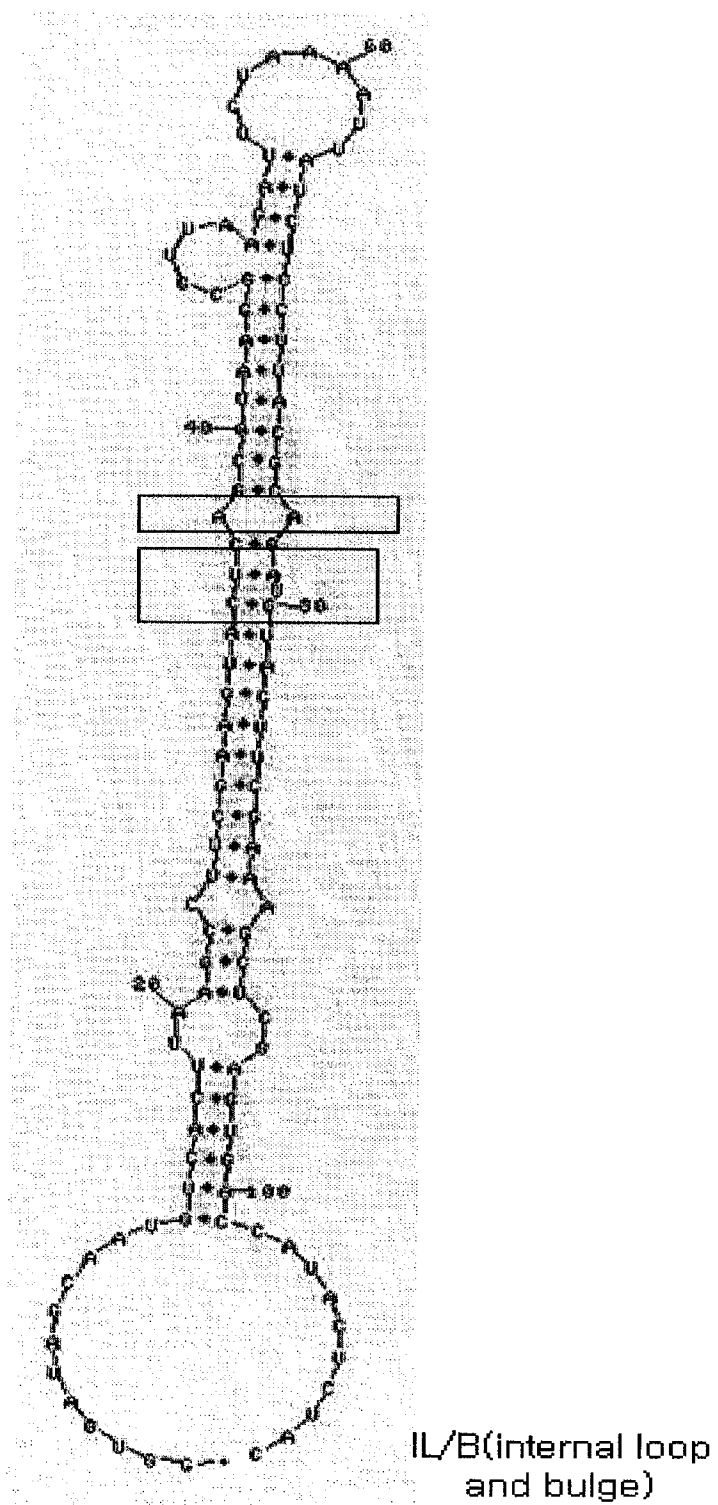
29/32

Fig. 30



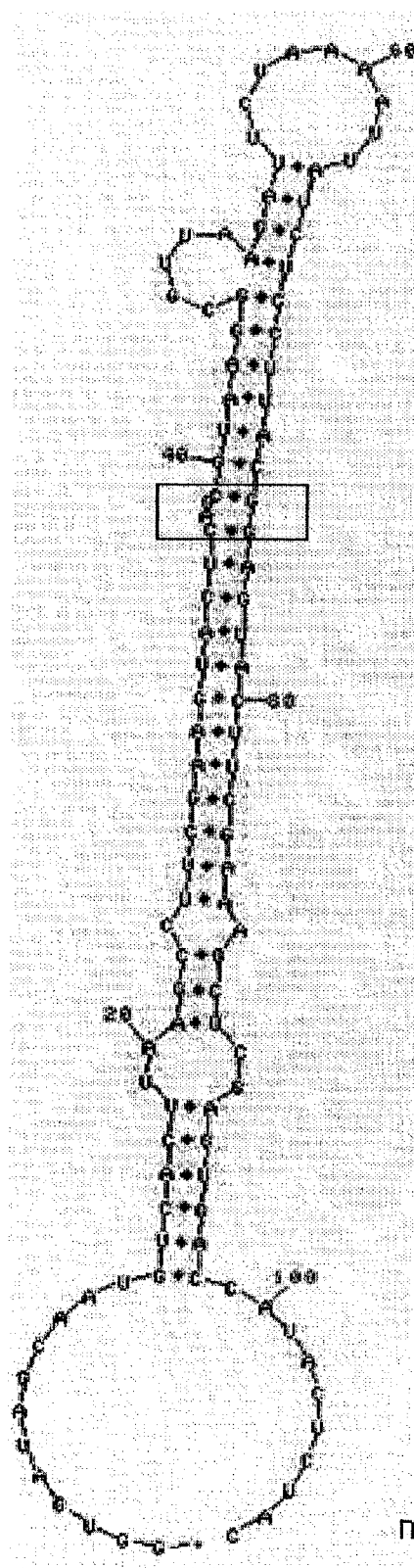
30/32

Fig. 31



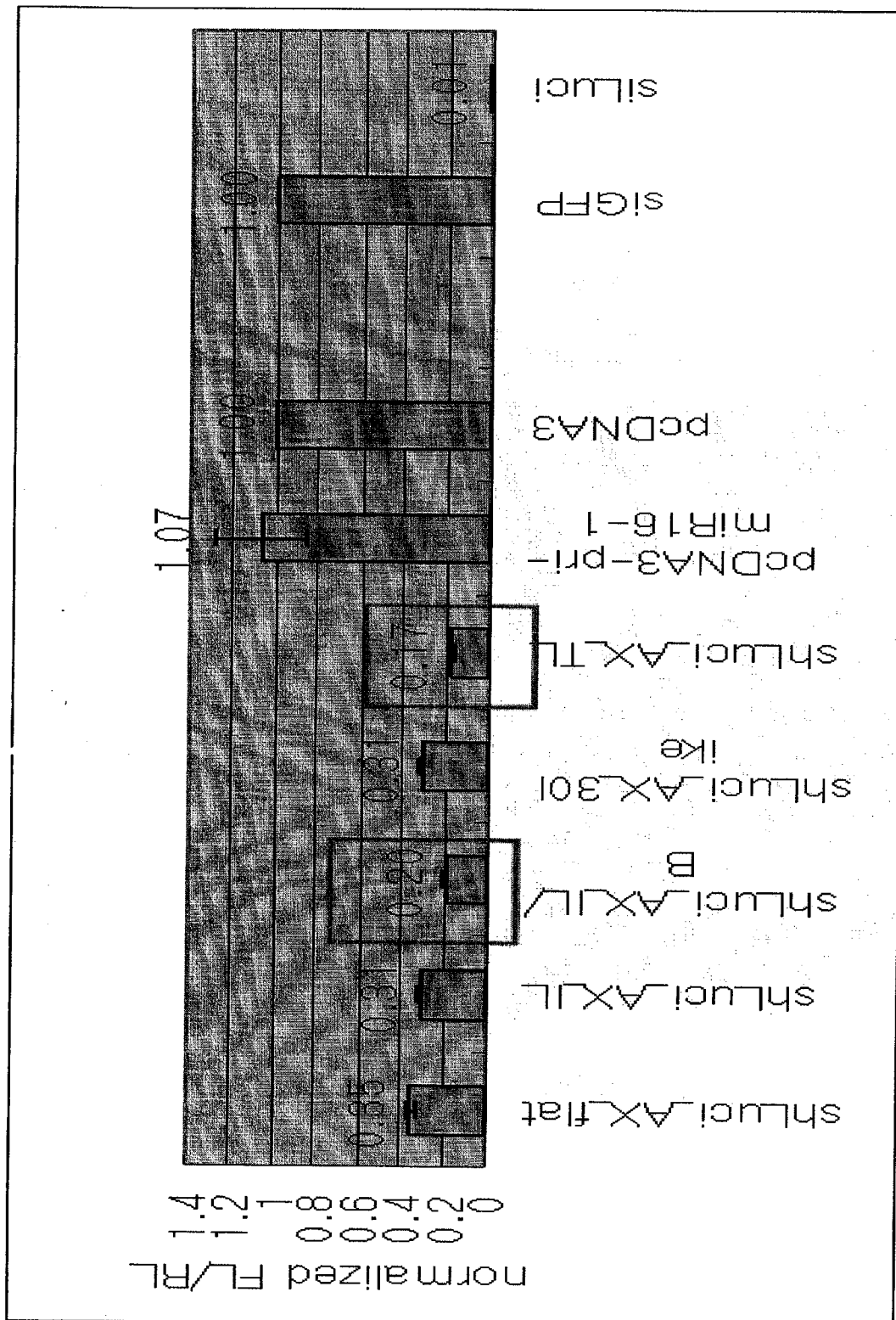
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Fig. 32



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Fig. 33



Sequence listing

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/002114**A. CLASSIFICATION OF SUBJECT MATTER***C12N 15/11(2006.01)i*

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)

IPC8: C12N 15/11

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

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

Esp@net, Pubmed, Delphion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZENG, Y. et al.: 'Recognition and cleavage of primary microRNA precursors by the nuclear processing enzyme Drosha' The EMBO Journal, Vol. 24(1), pp. 138-148 (25 November 2005) Fig. 9; p. 145, right-hand column, lines 54-55; p. 147, left-hand column, lines 28-32, 44-48.	1-51
A	DICKINS, R. A. et al.: 'Probing tumor phenotypes using stable and regulated synthetic microRNA precursors' Nature Genetics, Vol. 37(11), pp. 1289-1295 (2 October 2005) See the whole document.	1-51
A	NAM, J. W. et al.: 'Human microRNA prediction through a probabilistic co-learning model of sequence and structure' Nucleic Acids Research, Vol. 33(11), pp. 3570-3581 (24 June 2005) See the whole document.	1-51
A	LEE, Y. et al.: 'MicroRNA maturation: stepwise processing and subcellular localization' The EMBO Journal, Vol. 21(17), pp. 4663-4670 (2 September 2002) See the Abstract.	1-51



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

"E" earlier application or patent but published on or after the international filing date

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"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

21 FEBRUARY 2007 (21.02.2007)

Date of mailing of the international search report

22 FEBRUARY 2007 (22.02.2007)

Name and mailing address of the ISA/KR

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Authorized officer

KIN, Ji Yun

Telephone No. 82-42-481-8288



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2006/002114

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CAI, X. et al.: 'Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs' RNA, Vol. 10(12), pp. 1957-1966 (3 November 2004) See the Abstract.	1-51

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2006/002114

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

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

a. type of material



a sequence listing



table(s) related to the sequence listing

b. format of material



on paper



in electronic form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in electronic form



furnished subsequently to this Authority for the purposes of search

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

3. Additional comments: