

# Target prediction of candidate mirnas



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## **Candidate miRNA target prediction**

In silico target prediction of candidate miRNAs were conducted through TargetScanFly6 (Kheradpour et al., 2007), with a version last updated in 2015. The top three non-hypothetical proteins listed are considered top hits; however, if a gene within sight is known to be directly related to cellular localization or biogenesis and is highly expressed in reproductive tissue i. e. ovaries or testes, it is included over another gene which show neither of those characteristics, despite being a more highly ranked target. Gene ontology details and RNA-seq data were retrieved from publicly available information on FlyBase (Gelbart et al., 1997; Gelbart & Emmert, 2013). Target mRNA-specific primer pairs were designed on the Primer3 platform or through NCBI's own primer-pick function. These are named accordingly, with the acronym of its candidate miRNA preceding the gene, e. g. m932-geneX. Another function of TargetScanFly i. e. ORF was used to corroborate predictions, by introducing the 3'UTR sequence of CTPsyn (CG6854) into the software.

## **Generating crosses and phenotyping**

Germline miRX-overexpression was enabled by generating crosses of nos-GAL4 to the following lines: (a) UASp-miR190 lines i. e. 116443 from Kyoto and #117 from IMCB Singapore (courtesy of the Cohen lab), and (b) UASp-miR932 lines i. e. 116480 from Kyoto and #36 from IMCB. No UASp-bearing constructs are available for both miR-975 and miR-1014. MiRNA-sponges were driven by Act5c-GAL4 and T-155-GAL4. A miR971-sponge was included as a secondary control for the purpose of comparison, and to exclude sponge-induced phenotypes. Overexpression of candidate miRNAs were

repeated with crossings to Act5c-GAL4 flies. Testes of 3 to 5 DAE males obtained were immune-stained against CTPsyn according to the protocol described in Section . The behavioural traits of cytophidia i. e. localization, length, and numbers were determined under a confocal microscope (Zeiss LSM710 Platform, Germany). Genotypic details of fly lines mentioned here are listed in Table.

Table: Fly lines utilized for third phase of screening. The main construct carried by each line is in bold.

Driver Fly line # Genotype

nos-GAL4 116443 w[\*]; M{w[+mC]= UASp-mir-190. S}

elbZH-86Fb / TM3, Sb[1]

#117 UASp-miR-190@86Fb

116480 w[\*]; P{y[+t7. 7] w[+mC]= UASp-mir-932. S}attP16 / CyO

#36 UASp-miR-932@attp2

T155-GAL4 and Act5c-GAL4 61397 w[\*]; P{y[+t7. 7] w[+mC]= UAS-mCherry. mir-190. sponge. V2}attP40; P{y[+t7. 7] w[+mC]= UAS-mCherry. mir-190. sponge. V2}attP2

61439 w[\*]; P{y[+t7. 7] w[+mC]= UAS-mCherry. mir-932. sponge. V2}attP40; P{y[+t7. 7] w[+mC]= UAS-mCherry. mir-932. sponge. V2}attP2

63044 w[\*]; P{y[+t7. 7] w[+mC]= UAS-mCherry. mir-975. sponge. V2}attP40; P{y[+t7. 7] w[+mC]= UAS-mCherry. mir-975. sponge. V2}attP2

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61497 w[\*]; P{y[+t7. 7] w[+mC]= UAS-mCherry. mir-1014. sponge.

V2}attP40/CyO; P{y[+t7. 7] w[+mC]= UAS-mCherry. mir-1014. sponge.

V2}attP2

61457 w[\*]; P{y[+t7. 7] w[+mC]= UAS-mCherry. mir-971. sponge.

V2}attP40/CyO; P{y[+t7. 7] w[+mC]= UAS-mCherry. mir-971. sponge.

V2}attP2

### Designed constructs and properties

Primers flanking the target region were used in standard PCR. Amplified product was purified on-column (PCR and Gel Purification Kit, GeneXpress, Malaysia) and sent for sequencing to confirm the successful incorporation of desired sequences. Properties and functions of the 16 constructs generated were as follows: (A) Five of pTub-eGFP-miRX; the plasmid encodes for green fluorescent protein (GFP). Oligonucleotide sequences complementary to a particular candidate-miRNA was ligated to the end of the GFP gene. These therefore function as sensors for endogenous miRNA expression levels; (B) Five pAc-360-miRXov; for overexpression of candidate miRNAs. A ~200bp sequence encompassing the pre-miRNA of candidates flanked by 75bp either way. The ubiquitous promoter Act5c immediately upstream to it will induce a constant expression of the miRNA; (C) pTub-eGFP-3'UTR-IsoC; the 228bp 3' UTR stretch of CTPsynIsoC mRNA was attached to the end of the GFP gene. This construct is to be concurrently transfected with overexpression plasmids; (D) pAc-360-linker; as site for tethering of multiple units of miRNA-binding site (MBS) oligonucleotides, and finally; (E) Four of pAc-360-linker-miRXsp; ~50bp oligonucleotides tethered to one another within the SanDI

linker site of (D) construct. These are designed to act as alternative sites of elicitation for candidate-miRNAs as they are expressed endogenously, thus ‘sponging’ them from the cell’s cytoplasm and silencing their regulatory abilities. Schematic representations of each construct.

Constructs generated for in vitro assays. (A) miRX-sensors are essentially sequences complementary to the mature miRX, tethered to the end of a reporter gene, which in this case is eGFP. If a miRNA is highly present, its binding to eGFP-mRNA will lead to its degradation, and therefore lower levels of detected GFP-positive cells in flow cytometer. (B) miRX-overexpression construct, designed to induce expression of miRNAs. (C) The 3’UTR of CTPsynlsoC is attached to the very end of the eGFP gene; if a miRNA candidate directly elicits the region, it will attenuate GFP detection in much the same way as described for miRX-sensors. (D) The pAc-360 backbone is introduced with a linker region with a *SanDI* RE site. In (E) this RE is used to break the linker region free, upon which MBS designed to be flanked by their own *SanDI*-sites could be incorporated within, creating a construct which carries multiple copies of complementary binding sequences of miRX, thusly creating a sponge-like effect.

### **Transfection protocol**

Effectene® (Qiagen, Hilden, Germany) was used in DNA transfection into S2 cells, based on numerous studies reporting the effectiveness of this non-liposomal system over Lipofectamine© for insect cell cultures in general. Cells were seeded into treated plates in the recommended density of  $4 \times 10^5$  cells/ml. After 24 hours, plasmids were introduced according to the manufacturer’s protocol. Enhancer®, the accompanying reagent to

Effectene<sup>®</sup>, was kept at a consistent 8: 1 ratio to total amount of plasmid DNA to be transfected into cells. Based on a cytotoxicity test conducted preceding mass transfection, the maximum volume of Effectene<sup>®</sup> applicable to a 6-well plate was 15 $\mu$ l.