RNAi for Zebrafish

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Design and Use of shRNAs in Zebrafish

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***See also De Rienzo, Gutzman and Sive, (July 2012, in press) Efficient shRNAmediated inhibition of Gene Expression in Zebrafish. Zebrafish, Open Access.**http://online.liebertpub.com/doi/abs/10.1089/zeb.2012.0770

Background:
shRNAs (short hairpin RNAs) are processed into short interfering RNAs (siRNAs) that can inhibit gene expression (Fig.1). Using a recently described procedure for zebrafish (Dong et al, 2009), the miR30 gene transcribed region can be used to efficiently produce RNA hairpins, where the normal miR30 hairpin is replaced with a hairpin targeting a gene of interest (GOI) (1). Hairpins are then processed into interfering RNA. In general, it is most efficient to express the shRNA from a promoter. Where the guide strand and target are exact matches, the target RNA is cleaved and subsequently degraded by endogenous nucleases.

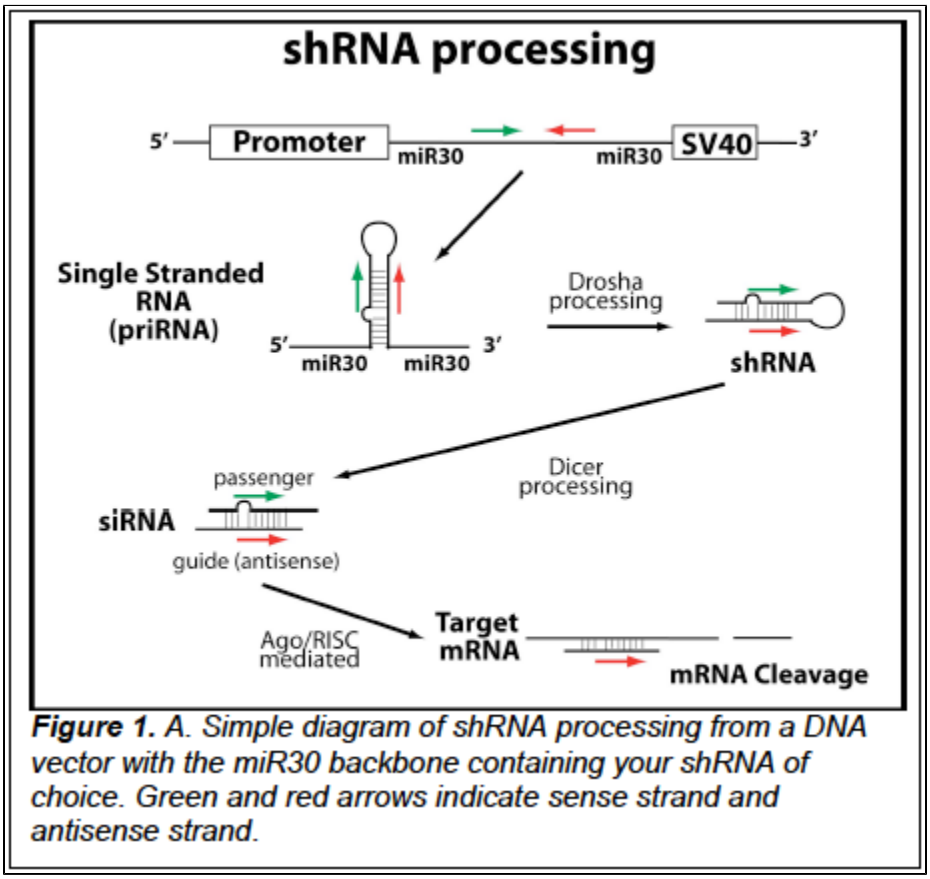

***Figure 1.** A. Simple diagram of shRNA processing from a DNA vector with the miR30 backbone containing your shRNA of choice. Green and red arrows indicate sense strand and antisense strand.*

Proceed as follows.

I. Design a hairpin to your gene of interest (GOI). This will be part of a dsDNA molecule that will be inserted into the miR30 backbone.
(see Detailed Protocols Section I: RNAi Hairpin Design)

II. Clone your GOI hairpin into the miR30 backbone. This step can be done either by
a. ordering two complementary, purified oligos, anneal these and subclone into the cut vector. Such oligos run~$80 apiece, for a total of ~$160 per test hairpin.

b. ordering unpurified oligos, for ~$30 each (total ~$60) anneal and purify these yourself to save on cost

(see Detailed Protocols Section III: Oligo Purification/oligo annealing, phosphorylation and ligation protocols below.)

III. Place the miR30 backbone behind a ubiquitous or a tissue-specific promoter and a fluorescent reporter, which can be transcribed from the same promoter. In Fig. 2 Construct A, contains the promoter upstream of the fluorescent protein followed by the zebrafish miR30 backbone, and of the SV40 poly A site. With this DNA construct, expression of GFP reflects transcription of the shRNA. Alternatively, as in Fig. 2 Construct B, the miR30 backbone has been placed in the intron of the actinexon-intron-fluorescent protein construct described by Dong et al. 2009 (1). After transcription, correct splicing places the actin ATG in frame with the fluorescent tag; therefore, expression of the tag indirectly indicates RNAi expression.

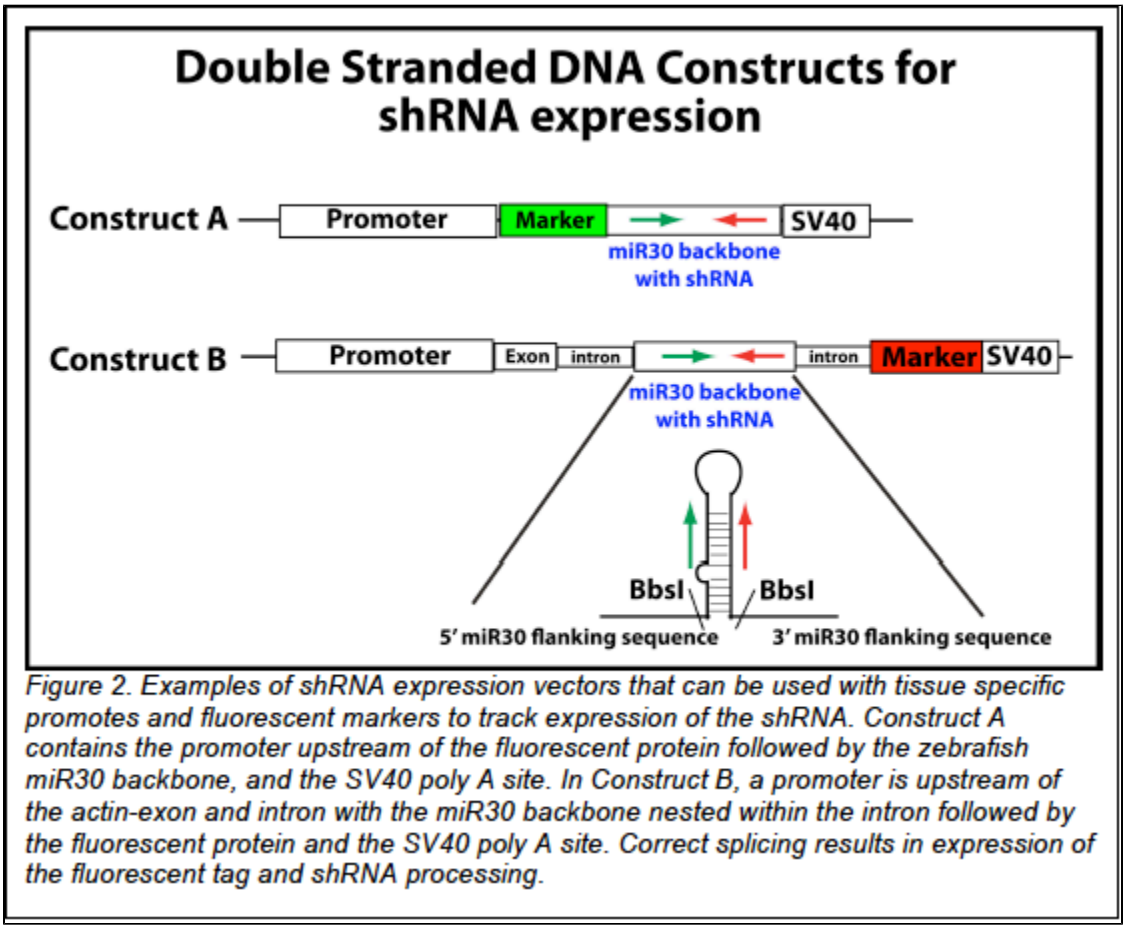

Figure 2. Examples of shRNA expression vectors that can be used with tissue specific promotes and fluorescent markers to track expression of the shRNA. Construct A contains the promoter upstream of the fluorescent protein followed by the zebrafish miR30 backbone, and the SV40 poly A site. In Construct B, a promoter is upstream of the actin-exon and intron with the miR30 backbone nested within the intron followed by the fluorescent protein and the SV40 poly A site. Correct splicing results in expression of the fluorescent tag and shRNA processing.

IV. Express the construct as a transgenic, usually initially, an F0 transgenic. Transgenesis can be effected by meganuclease (I-SceI) or Tol2 depending on the vector backbone.
Monitor endogenous target RNA decrease using qPCR and in situ hybridization. Monitor phenotypes and possible rescue by expression/injection of target RNA not subject to siRNA inhibition. **(see protocols section IV.)**

An easy way to allow interchanging of promoters and is through use of the Gateway system (Fig. 3) (2,3). Transgenesis is generally obtained by Tol2 methods.

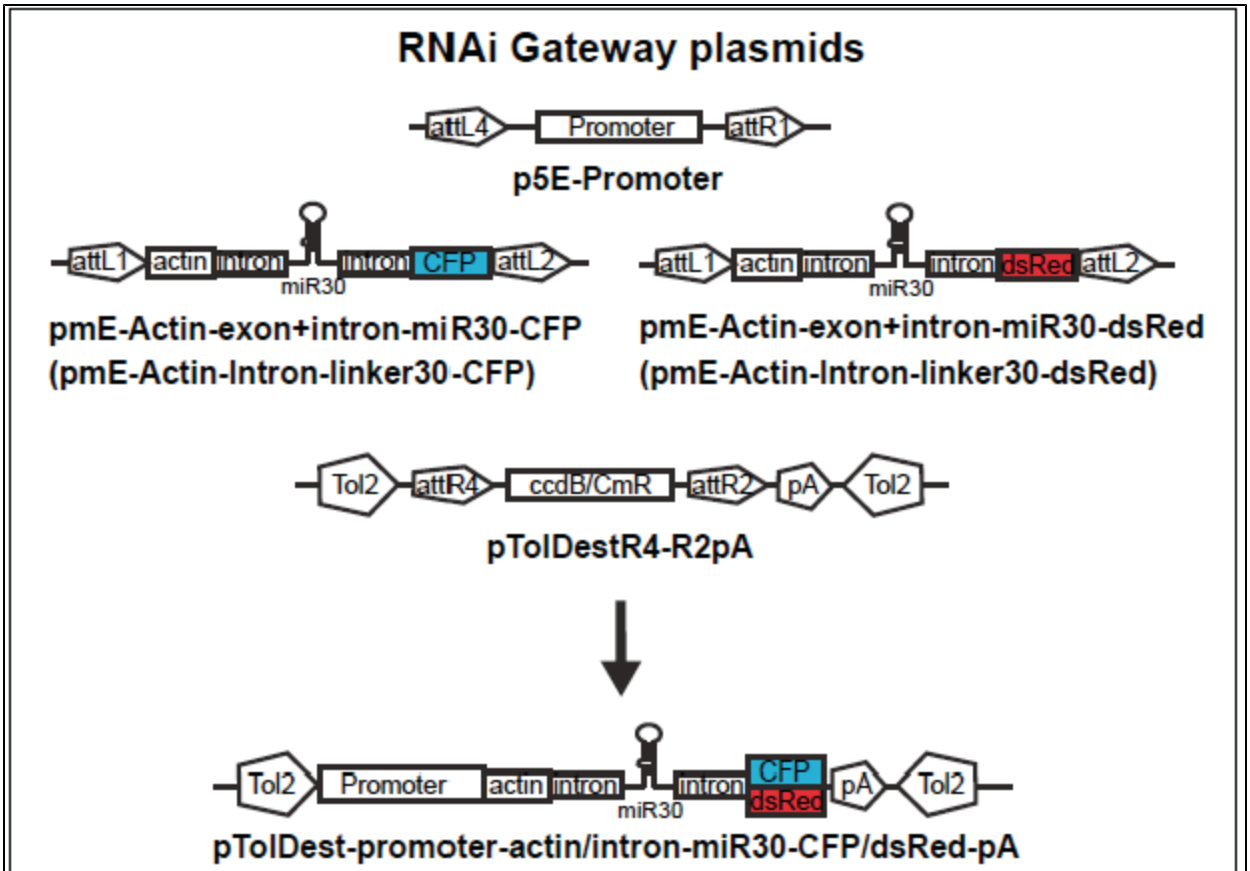

Figure 3. RNAi Gateway plasmids. The promoter of interest is cloned into the 5' element (p5E-Promoter). The actin-exon-intron-miR30-fluorescent protein containing the hairpin of interest is cloned into the middle element plasmid (pme-MCF) to yield pmE-Actin-exon+intron-miR30-CFP/dsRed, also called pmE-Actin-intron-linker30CFP/dsRed. DNA encoding shRNAs is inserted into the miR30 backbone contained within these pmE plasmids (available on request). The 5' and the middle element plasmids are assembled together with the destination vector (pTolDestR4-R2pA) to create the expression vector pTolDest-promoter-actin/intron-miR30-CFP/dsRed-pA.

DETAILED PROTOCOLS

I. RNAi hairpin Design

The protocol below describes design and cloning of oligos containing a hairpin that will be inserted into a miR30 backbone.

1. Use the Invitrogen Block-IT RNAi designer website for finding shRNA sequences that would target your gene of interest.

2. Use this link and choose to design an shRNA, then enter your sequence or accession number.https://rnaidesigner.invitrogen.com/rnaiexpress/setOption.do?designOption=shrna&pid=- (https://rnaidesigner.invitrogen.com/rnaiexpress/setOption.do?designOption=shrna&pid=\-) 248931025050769267

3. Choose hits that are 4-5 stars and blast the sequences to the zebrafish genome to be sure your target gene is your only hit (anything else should be looked at more carefully since off target effects are possible depending on your target sequence and region of genome match within the hairpin).

4. Your shRNA target sequence should be a 21 mer.

5. Design 4 – 6 shRNAs per target. These can be in the coding sequence, or 3'UTR (De Rienzo et al, 2012).

6. Follow the example below to add in your sequence for designing your RNAi oligo. (should be a 71 mer).

7. YFP shRNA design example

Enhanced YFP NCBI ACCESSION: DI026212

1 atggtgagca agggcgagga gctgttcacc ggggtggtgc ccatcctggt cgagctggac
61 ggcgacgtaa acggccacaa gttcagcgtg tccggcgagg gcgagggcga tgccacctac
121 ggcaagctga ccctgaagtt catctgcacc accggcaagc tgcccgtgcc ctggcccacc
181 ctcgtgacta ccttcggcta cggcctgatg tgcttcgccc gctaccccga ccacatgaag
241 cagcacgact tcttcaagtc cgccatgccc gaaggctacg tccaggagcg caccatcttc
301 ttcaaggacg acggcaacta caagacccgc gccgaggtga agttcgaggg cgacaccctg
361 gtgaaccgca tcgagctgaa gggcatcgac ttcaaggagg acggcaacat cctggggcac
421 aagctggagt acaactacgg tggatccggt gctagcaaca gccacaacgt ctatatcatg
481 gccgacaagc agaagaacgg catcaaggtg aacttcaaga tccgccacaa catcgaggac
541 ggcagcgtgc agctcgccga ccactaccag cagaacaccc ccatcggcga cggccccgtg
601 ctgctgcccg acaaccacta cctgagctac cagtccgccc tgagcaaaga ccccaacgag
661 aagcgcgatc acatggtcct gctggagttc gtgaccgccg ccgggatcac tctcggcatg
721 gacgagctgt acaagtaa

pink = 21 mer Invitrogen Block-IT design 5 stars (THIS HAS NO BLAST HITS IN THE ZEBRAFISH GENOME)

8. Oligo design with color coding (YFP example, continued)

YFP_RNAi_Forward oligo

5'GGCTAGCAGCCACAACGTTTCTATATCATGGCTGGTGCACATGATGGAGCCATGATATAGACGTTGTGGCC –3'

3' TCGTCGGTGTTGCAAAGATATAGTACCGACCACGTGTACTACCTCGGTACTATATCTGCAACACCGGTCGG –5'

YPF_RNAi_Reverse oligo

Underlined letters are overhang for the BbsI sticky ended subcloning with the miR30 backbone construct.

grey is an Nhe1 site that is introduced only on ONE side for digest confirmation of your construct. (You can also design the restriction site of your choice in this region.)

The two TT at nucleotide 18 and 19 are the di-nucleotide bulge on the passenger strand which help prevent nonspecific cellular responses. (Bauer et al 2009).

Green are the14 bases in the loop from the mir30 backbone, keep this sequence the same.

red letters are the antisense sequence of your hairpin, a perfect match to your DNA sequence.

blue letters are the sense strand of your hairpin.

****NOTES:**
All parts of the construct stay the same for each new targeting sequence EXCEPT FOR THE RED AND BLUE LETTERS. Be sure to properly create your 3' to 5' compliment.
When ordering, remember that the two oligos above, that you anneal, are slightly different because of the NheI restriction site only on one side so you cannot have the program do an automatic reverse compliment.

II. Oligo Urea-PAGE Purification Protocol

1. Pour a large (~6inch X 6inch) denaturing UREA – PAGE gel.
% of your gel depends on the oligo size.
70 mer 8-10%
using National diagnostics reagents.

No. EL-830
835 {order EL-833 (kit with all three)
840
The gel thickness depends on the amount you need to purify.
You will get a band at the correct size – then a smear of unpure oligo below your correct size band. For single nucleotide resolution decrease your starting material and run the gel longer.

2. Resuspend lyophilized oligo in H2O
Add 100 l H2O to 50nmol of oligo and resuspend oligo by mixing at room temperature.
take about 50-20 l oligo in H2O
add 2x loading dye
* Use loading dye containing both bromophenol blue and xylene cyanol with 8 M urea.
RECIPES

Urea Gel Loading Dye
2X mix is:
8M Urea (FW 60.06)
25mM EDTA (FW 372.2)
Use:
Urea: RSB gold capped molecular biology grade (cat. US 75826 1kg)
EDTA: Sigma molecular biology grade (cat. E-5134 500g)
Make:
1000mL, RNase-free (use no glass containers). Use only weigh boats from middle of stack and only plastic containers from Corning 0.22um filter apparatus. You will need 2 500mL filter apparatuses and 2 1000mL apparatuses (one to mix and one to actually filter).
Prepare 500mL, 500mM EDTA stock at pH 8.0 (see Maniatis 3 B.11)
a. to 93.1g EDTA, add 10g NaOH pellets; dump into 500mL filter bottle.
b. add nuclease-free H2O to 500mL
c. mix on orbital shaker until dissolved (overnight)
d. filter
Dissolve urea:
a. add 480g urea to 1000mL filter bottle
b. add nuclease-free H2O to 900mL
c. mix on orbital shaker until dissolved (overnight)
d. add 50mL, 500mM EDTA
e. top-off to 1000mL with nuclease-free H2O
f. filter
Preparing dyes to aliquot: we use 0.025% Xylene Cyanol and Bromophenol Blue, so weigh out 0.0125g per 50mL.
3. Load/Run gel with 0.5 x TBE running buffer
Start @ 2 watts 10-15 mins
Increase to 10-15 watts
Check the xylene dye front to determine how far to run your gel
Xylene runs at 55 bp with 8% gel

4. When finished take gel apart and put the gel between 2 pieces of saran wrap.
5. Look at the gel with UV shadowing over fluor-coated TLC plate.
Plates available from Applied Biosystems AM10110 10x10cm ~$50
Put saran wrapped gel on the plate – Quickly scan with UV light – Look for shadows. (The shadows are your bands and indicate the presence of DNA blocking the UV light)
Draw on the saran wrap around Shadow with Sharpie (do Not cut your gel on the Plate)

6. Cut out gel pieces and put the gel containing your oligo in an eppendorf tube.
Cut into pieces if necessary –
Want gel piece about ~1square centimeter (too big will lead to impure oligo!) You do not want to include any of the smear below your pure large band.

7. Add 400 l 0.3M NaCl in H2O to tube (400 l/tube of gel).
Rotate overnight @ 4°C.
DNA will come out of gel into salt!

8. Spin tube – acrylamide piece will be at bottom.
Collect Supernatant = oligo.

9. Add 2.5 x volumes of ethanol
2.5 x volumes 400ul. (so 1 ml for 400 l)
into -20°C at least 2 hrs.

10. Spin on high --
oligo= pellet! (10 min @4°C)
Dry pellet!
Resuspend in 50l nuclease free water and spec for your actual DNA oligo concentration

11. Use for annealing / oligo phosphorylation/ ligations (see protocols below).
****NOTES:**

Should recover 25% of amount of oligo loaded on gel.
For example, based on starting material of ~ 660 g loaded
Predict with 25% recovery = ~ 150 g
Add 50 l nuclease free water to each tube
Do spec reading
If see a range of 900 ng/l – 2500 ng/l, this means good recovery.

III. Oligo annealing, phosphorylation and ligation protocol

Following oligo purification, oligos are annealed, checked, phosphorylated and used as insert for ligation your specific promoter driven vector of choice with the miR30 backbone and a fluorescent tag.

Annealing

Following oligo purification, oligos are annealed in a 50l reaction
1l of 100M forward oligo
1l of 100M reverse oligo
25l of annealing buffer
23l of water
TOTAL 50l
Annealing buffer: 200mM postassium acetate, 60mM HEPES-KOH pH 7.4, 4mM magnesium acetate

(Another option for re-suspension of purified oligos and use as an annealing buffer:
10 mM Tris, pH 7.5--8.0, 50 mM NaCl, 1 mM EDTA)

For the annealing reaction:
Use a PCR machine, run the following program:
4’ 95°C
10’ 70°C
2’ 60°C
2’ 50°C
2’ 40°C
2’ 30°C
2’ 20°C
2’ 10°C
store at 4°C before use

Check for oligo annealing by running your annealed oligos, compared to single oligos, on a 2% DNA agarose gel. The annealed oligos will run slower than the singles.

Oligo Phosphorylation
Annealed oligos 400 ng
10X ligation buffer (supplied with 10mM ATP) 3 l
10 U/l T4 polynucleotide kinase 2 l
H20 to 30 l
Incubate at 37°C for 30 minutes. Use directly for the ligation reaction.
Final concentration of the phosphorylated annealed oligo pair: ~13 ng/l

Ligation reaction
Combine vector (containing miR30 backbone with BbsI sites) and the insert (annealed oligo) at a molar ratio of 1:5

Prior to ligation, de-phosphorylate your vector using Antarctic Phosphatase treatment (New England Biolabs).

Ligation reaction example:

vector (length 4479)	100 ng
insert (length 68)	7.6 ng
10X ligase buffer	2 l
400U/l ligase	1 l
Water	X l

TOTAL 20l reaction
Incubate
12 h at 15°C

Transformation
Transform DH5 cells with 5 l of the ligation reaction
30’ on ice
5’ at 42°C
5’ on ice
add 300 l of SOC media

1 h at 37°C
Plate with appropriate antibiotic

IV. Use of shRNAs in transient transgenic assays

Transgenic preparation
Initial analysis can be performed in transient (F0) transgenics.

For Tol2 vectors:
Combine:

125 ng/l transgenic plasmid	4 l
125 ng/l Tol2 mRNA	4 l
H20	12 l

Inject 1nl of the solution into one-cell-stage eggs.
The mass amount of plasmid and transposase injected per embryo will be 25 pg of each.

Note
We found that this concentration is the best ratio for most of the constructs giving high fluorescence and no necrosis. To increase fluorescence, it is possible to titrate the DNA up to 70 pg but it will increase necrosis. To avoid necrosis it is possible to coinject 3 ng of p53MO (4).
Expect between 20-50% of dead/deformed embryos as a sign that the transposase is active and transposition is occurring.

For meganuclease vectors:
Combine

150 ng/l transgenic plasmid	4 l
5 U/l ISce-I	2 l
10X ISce-I buffer	2 l
H20	12 l

Inject 1nl of the solution into one-cell-stage eggs
The concentration of plasmid/meganuclease injected per embryo will be 30 pg/0.5 U

Note
We found that this concentration is the best ratio for most of the constructs giving high fluorescence and no necrosis. To increase fluorescence it is possible to titrate the DNA up to 70 pg but it will increase necrosis.

Subsequent steps
• Score embryos for fluorescent protein expression.
• Quantify gene knockdown by qPCR.
• Score fluorescent embryos for phenotype.
• Attempt rescue of phenotype by injection or expression of target gene RNA that does not bind siRNA.

REFERENCES
1. Dong M, Fu YF, Du TT, Jing CB, Fu CT, Chen Y, Jin Y, Deng M, Liu TX. Heritable and lineagespecific gene knockdown in zebrafish embryo. PloS one. 2009;**4**, e6125.
2. De Rienzo G, Gutzman J, Sive H. Efficient shRNA-mediated inhibition of Gene Expression in Zebrafish. Zebrafish. July 2012. http://online.liebertpub.com/doi/abs/10.1089/zeb.2012.0770
3. Villefranc JA, Amigo J, Lawson ND. Gateway compatible vectors for analysis of gene function in the zebrafish. Dev Dyn. 2007;**236**:3077-3087.
4. Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, Parant JM, Yost HJ, Kanki JP, Chien CB. The Tol2kit: A multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. Dev Dyn. 2007;**236**:3088-3099.
5. Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, Farber SA, Ekker SC. p53 activation by knockdown technologies. PLoS Genet. 2007;**3**:e78.