File S1
Supplemental Methods

Construction of U6-based sgRNA expression vectors

1. Order a pair of U6 vector-specific, 23nt targeting primers for each target. For each target (GN19), order a forward primer TTCGN19 for the U6 promoter vectors; order a reverse primer AAACN19. Note that the N19 in the reverse primers is reverse complementary to that in the forward primer.

   To order primers compatible with both the pU6x-sgRNA vectors and pT7-gRNA (Addgene plasmid #4675; (JAO *et al.* 2013)), order degenerate primers WTMGGN18 (forward) and AMMCN_{18}C (reverse), where M=A or C, W=A or T. Again, the N18 in the reverse primers is reverse complementary to that in the forward primer.

2. Anneal the two primers (1µl 100µM stock each in a 20µl 1x NEB buffer 2) by incubating the mixture at 95^{o}C for 5 min, ramping down to 50^{o}C at 0.1^{o}C/sec, incubating at 50^{o}C for 10 min, and chilling to 4^{o}C at normal ramp speed.

3. Ligate the annealed oligos to the U6 vector of choice (pU6x-sgRNA #1-#5, see diagrams and plasmid list in the next pages) by mixing the components [1 µl 10x CutSmart buffer, 1 µl T4 DNA ligase buffer, 0.25µl U6 plasmid (about 100ng), 1µl annealed oligos, 0.3 µl T4 DNA ligase, 0.3 µl BsmBI, 0.2µl PstI (optional), 0.2 µl SalI (optional), 5µl H2O] and incubating 3x(37^{o}C for 20 min, 16^{o}C for 15 min), then 37^{o}C for 10 min, 55^{o}C for 15 min, followed by 80^{o}C for 15 min (optional).

4. The reaction is ready for transformation (use 2 µl of the ligation and plate 10% of the transformants). Transform and spread onto spectinomycin (50µg/ml) plates.

Construction of pGGDestTol2LC-sgRNA vectors

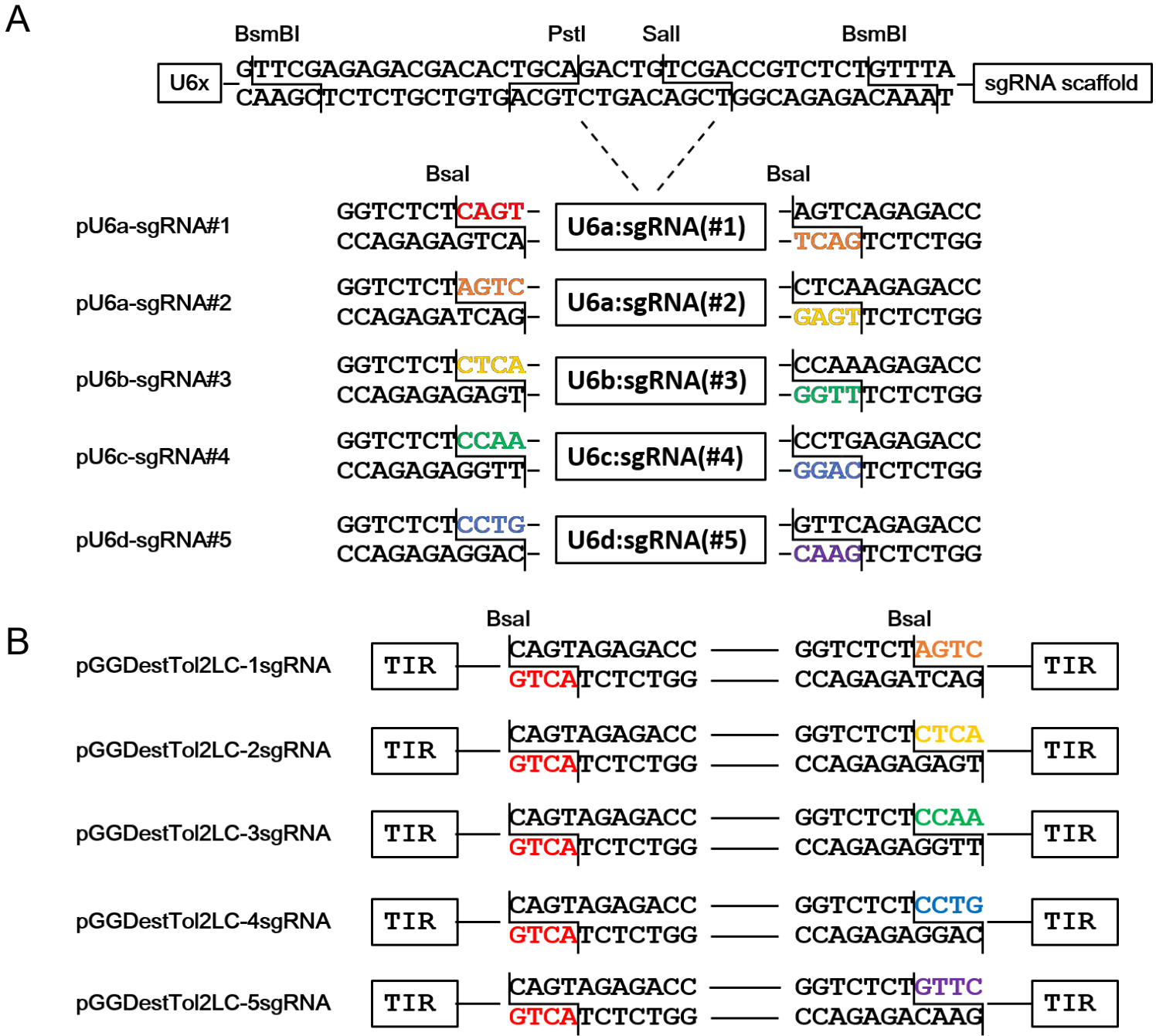
1. Ligate the U6x:sgRNA cassettes into a pGGDestTol2LC vector by mixing the components (2µl 10x CutSmart buffer, 2 µl T4 DNA ligase buffer, 100ng of each pU6x-sgRNA plasmid, 50ng empty pGGDestTol2LC-sgRNA vector of choice (see plasmid list and diagrams), 1µl BsaI, 1µl T4 DNA ligase, adjust volume to 20 µl with H2O).

2. Incubate mixture 3x(37^{o}C for 20 min, 16^{o}C for 15 min), followed by 80^{o}C for 15 min.

3. The reaction is ready for transformation (use 5 µl of the ligation and plate 50% of the transformants). Transform and spread onto ampicillin (100 µg/ml) plates.

Reference.

JAO, L. E., S. R. WENTE and W. CHEN, 2013 Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. Proc Natl Acad Sci U S A **110:** 13904-13909.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PstI
SalI
BsmBI
U6x
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sgRNA scaffold
BsaI
BsaI
pU6a-sgRNA#1
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CCAGAGAGTCA
U6a:sgRNA(#1)
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TCAGTCTCTGG
pU6a-sgRNA#2
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GAGTTCTCTGG
pU6b-sgRNA#3
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CCAGAGAGAGT
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GGTCTCTCCAA
CCAGAGAGGTT
U6c:sgRNA(#4)
CCTGAGAGACC
GGACTCTCTGG
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GGTCTCTAGTC
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CCAGAGAGAGT
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pGGDestTol2LC-5sgRNA
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GGTCTCTGTTC
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TIR

List of plasmids deposited at Addgene (Deposit 71794)

Plasmid ID	Plasmid Name
64237	pME-Cas9
64239	pGGDestTol2LC-1sgRNA
64240	pGGDestTol2LC-2sgRNA
64241	pGGDestTol2LC-3sgRNA
64242	pGGDestTol2LC-4sgRNA
64243	pGGDestTol2LC-5sgRNA
64245	pU6a:sgRNA#1
64246	pU6a:sgRNA#2
64247	pU6b:sgRNA#3
64248	pU6c:sgRNA#4
64249	pU6d:sgRNA#5
64250	pU6a:sgRNA(tyr)