Construction of U6-based sgRNA expression vectors

Linlin Yin, Lisette A. Maddison, Mingyu Li, Nergis Kara, Matthew C. LaFave,Gaurav K. Varshney, Shawn M. Burgess, James G. Patton, and Wenbiao Chen

Abstract

This protocols is from:
Linlin Yin, et al. (2015) Multiplex Conditional Mutagenesis Using Transgenic Expression of Cas9 and sgRNAs. *Genetics* 200:431-441; doi:10.1534/genetics.115.176917

Please see the full manuscript for additional details.

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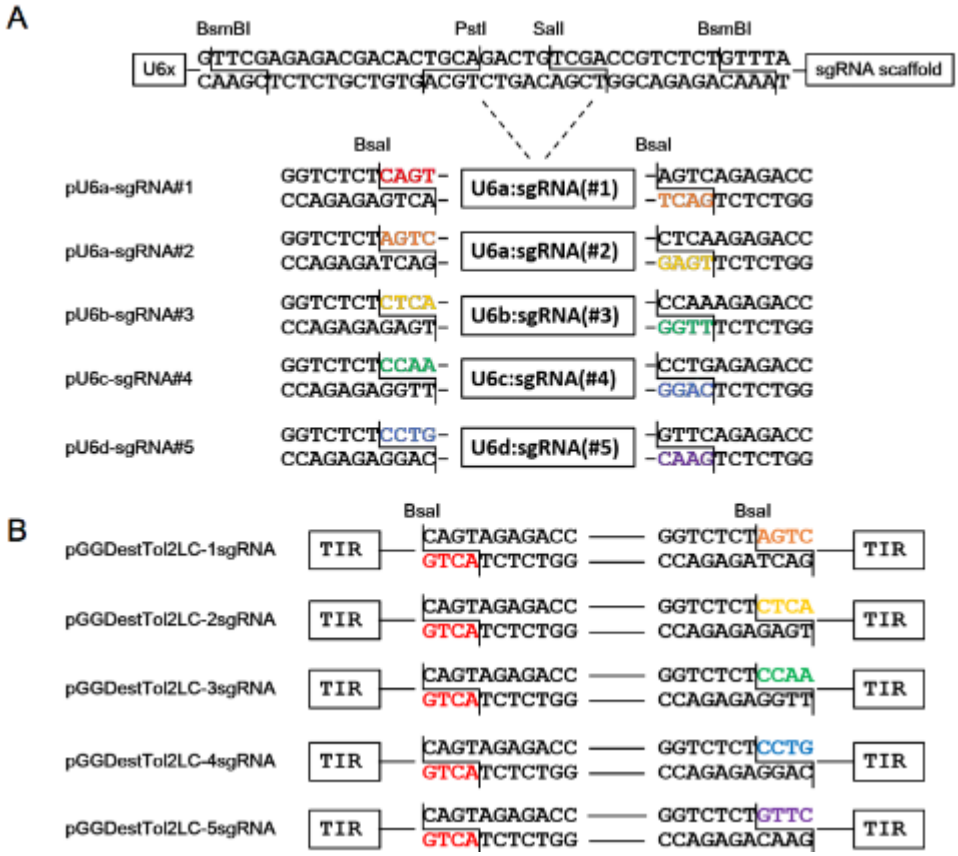
Guidelines



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)

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.

Protocol

Step 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 AMMCN18C (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

Annealing the primers

Step 2.
Mix 1µl 100µM stock each in a 20µl 1x NEB buffer 2

REAGENTS
NEBuffer 2 - 5.0 ml B7002S by New England Biolabs

Annealing the primers

Step 3.
Incubate the mixture as follows:

95 °C for 5 min,
ramp down to 50 °C at 0.1 °C/sec,
50 °C for 10 min
chill to 4 °C at normal ramp speed

Ligating the annealed oligos

Step 4.
Mix the following components to ligate the annealed oligos to the U6 vector of choice (pU6x-sgRNA #1-#5, see diagrams and plasmid list in the Guidelines)

PROTOCOL

. U6-based sgRNA ligation mixture

CONTACT: Tracey DePellegrin

Step 4.1.
1 µl 10x CutSmart buffer

Step 4.2.
1 µl T4 DNA ligase buffer

Step 4.3.
0.25µl U6 plasmid (about 100ng)

Step 4.4.
1µl annealed oligos

Step 4.5.
0.3 µl T4 DNA ligase

Step 4.6.
0.3 µl BsmBI

Step 4.7.
0.2µl PstI (optional)

Step 4.8.
0.2 µl SalI (optional)

Step 4.9.
5µl H2O

Ligating the annealed oligos

Step 5.
Incubate at 37 °C for 20 min

DURATION
00:20:00

Ligating the annealed oligos

Step 6.
Incubate at 16 °C for 15 min

DURATION
00:15:00

Ligating the annealed oligos

Step 7.
Incubate at 37 °C for 10 min

DURATION
00:10:00

Ligating the annealed oligos

Step 8.
Incubate at 55 °C for 15 min

DURATION
00:15:00

Ligating the annealed oligos

Step 9.

Incubate at 80 °C for 15 min (optional)

DURATION

00:15:00

Step 10.

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.