Molecular cloning of single CRISPR plasmid

Step 1: Order the following oligos:

Example target: 5′ ...CCGAGAAGGTTGGCTGGGTGCGG... 3′

Forward primer: cgtc[20nt protospacer]

Reverse primer: aaac[reverse complement of 20nt protospacer]

Order:

gRNA-TOP: 5′- cgtcCCGAGAAGGTTGGCTGGGTG - 3′

gRNA-BOT: 5′- aaacCACCCAGCCAACCTTCTCGG - 3′

EcoRI-HH-<Gene>-Fwd: 5′ – aaaGAATTC<NNNNNN>CTGATGAGTCCGTGAGGACG – 3′

This primer will be specific to each gRNA. <NNNNNN> is the reverse complement of the first 6 nucleotides of the gRNA protospacer. For the CCGAGAAGGTTGGCTGGGTG gRNA, this sequence will be <TCTCGG>, and the 'EcoRI-HH-<Gene>-Fwd' primer will be 5′-aaaGAATTCtctcggCTGATGAGTCCGTGAGGACG-3′.

The following primers are common for all constructs:

HDV-NotI-Rev: 5′ – aaagcggccgcGTCCCATTCGCCATGCCGAA – 3′

Citrine-ColonyPCR-Fwd: 5′ – CACAAGCTGGAGTACAACTA – 3′

T7-seq-Rev: 5′ – taatacgactcactataggg – 3′

Order and store 100μM/10μM dilutions at -20˚C.

Step 2: Set up the gRNA protospacer annealing reaction:

5 μL	100 µM gRNA TOP
5 μL	100 µM gRNA BOT
5 μL	NEB 10x T4 DNA Ligase Buffer
35 μL	Water
50 μL	Total volume

Heat to 95°C for 5 minutes. Bring to room temperature on the bench for 30-45 minutes.

Annealed oligos:

```
5′- cgtcCCGAGAAGGTTGGCTGGGTG     - 3′
        ||||||||||||||||||||
3′-     GGCTCTTCCAACCGACCCACcaaa - 5′
```

Dilute this reaction 1:1000. This is your insert.

Step 3: Digest the shuttle vector plasmid:

x μL	6μg of DNA
6 μL	10x Cutsmart Buffer
2 μL	BsaI-HF enzyme
(52-x) μL	Water
60μL	Total volume

Digest at 37˚C overnight (16-18 hours at least). Gel extract to obtain your shuttle vector. Do not CIP-treat. Ensure the vector concentration after elution is less than 50ng/μL (dilute if necessary).

Step 4: Ligate annealed oligos into the Shuttle vector:

1 μL	Linearized Shuttle vector
2.5 μL	gRNA annealed primers (1:1000 dilution from above)
2 μL	NEB 10x T4 DNA Ligase Buffer
1 μL	T4 DNA Ligase
13.5 μL	Water
20 μL	Total Volume

Incubate at room temperature for 20 minutes, then transform 25μL competent cells (*our lab uses homegrown competent cells*) with 5μL of ligation reaction (*This can be changed to maintain a 1:5 ratio of ligation mix to competent cells*). Add 37.5μL SOC medium (*1.5x volume of competent cells used*) and recover for 15 minutes at 37˚C. Plate full volume on LB+Amp plates. Incubate plates at 37˚C overnight.

(For first use after digesting the Shuttle vector, perform a "Shuttle vector only" negative control to test for digestion efficiency. Make sure you don't get any colonies. Repeat digestion if necessary.)

Step 5: Amplification of HH-gRNAf+e-HDV precursor:

Pick individual colonies and swirl in 10μL of SOC (in a PCR strip tube). Set up the following reactions:

Reaction 1

2.5 μL	Swirled Culture
5 μL	EcoRI-HH-<Gene>-Fwd
5 μL	HDV-NotI-Rev
2.5 μL	Taq Buffer
1 μL	dNTPs
1.5 μL	DMSO
0.5 μL	Taq Polymerase
32 μL	Water
50 μL	Total Volume

In parallel, set up the following colony PCR to verify successful integration of the protospacer:

Reaction 2

1 μL	Swirled Culture
2 μL	gRNA-TOP (10μM dilution)
2 μL	T7 seq Reverse
1 μL	Taq Buffer
0.4 μL	dNTPs
0.6 μL	DMSO
0.2 μL	Taq Polymerase
12.8 μL	Water
20 μL	Total Volume

Run 2μL of Reaction 1 and 20μL of Reaction 2 on a gel. Reaction 1 should yield a ~215bp product, while Reaction 2 should yield a 690bp product (if the protospacer was successfully integrated into the shuttle vector). PCR purify the remaining 48μL of Reaction 1 (for colonies that have a positive band for Reaction 2) and elute in 52μL. Set up the following digestion reaction:

52 μL	PCR-purified product
6 μL	10x Cutsmart Buffer
1 μL	EcoRI-HF enzyme
1 μL	NotI-HF enzyme
60μL	Total volume

Digest for 2 hours at 37˚C. Gel extract. This is your "EcoRI-HH-gRNA-HDV-NotI" insert.

Step 6: Clone HH-gRNAf+e-HDV precursor into single CRISPR plasmid:

Set up the following digestion reaction:

x μL	6μg of single CRISPR plasmid
6 μL	10x Cutsmart Buffer
1 μL	EcoRI-HF enzyme
1 μL	NotI-HF enzyme
(52-x) μL	Water
60μL	Total volume

Digest at 37˚C overnight. Gel extract to obtain the CRISPR destination vector. Set up ligation at 1:3 molar ratio using this vector and the insert obtained in Step 5.

x μL	Linearized CRISPR destination vector
y μL	Digested and purified HH-gRNA-HDV insert (from Step 5)
2 μL	NEB 10x T4 DNA Ligase Buffer
1 μL	T4 DNA Ligase
(20-x-y) μL	Water
20 μL	Total Volume

Ligate for 2 hours at room temperature. Transform 25μL competent cells (*our lab uses homegrown competent cells*) with 5μL of ligation reaction (*This can be changed to maintain a 1:5 ratio of ligation mix to competent cells*). Add 37.5μL SOC medium (*1.5x volume of competent cells used*) and recover for 1 hour at 37˚C. Plate and incubate at 37˚C overnight.

The next day, validate colonies by colony PCR using Citrine-ColonyPCR-Fwd and 10μM dilution of gRNA-BOT primers. Validate insert by sequencing and electroporate chick embryos at 2.5μg/μL concentration.