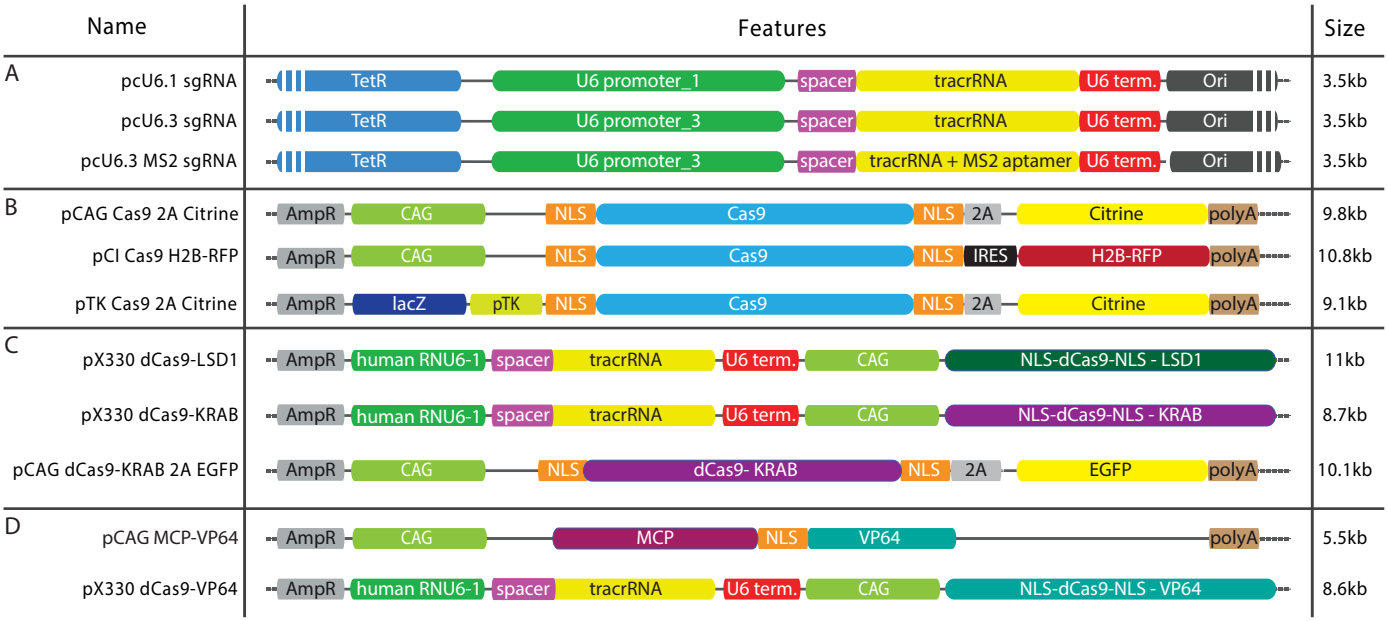


Figure S1. Novel chick Genome Engineering and Epigenome Engineering toolkit vectors. (A) pcU6 sgRNA expression mini-vectors containing U6.1 and U6.3 promoters are shown, as well as the pcU6.3_MS2 sgRNA expression vector incorporating MS2 stem loops as binding aptamers for bacteriophage MS2 coat proteins (MCP) used in the Synergistic Activation Mediator (SAM) method. All mini-vectors contain a BsmBI flanked spacer cloning site. (B) Two ubiquitous Cas9 plasmids, co-expressing 2A-Citrine or IRES-RFP reporter gene. pTK Cas9 construct was also generated to enable enhancer driven cell-type specific Cas9 expression. (C) dCas9-effector plasmids, dCas9-LSD1 and dCas9-KRAB were generated in pX330 backbone. dCas9-KRAB with EGFP reporter was cloned into the pCAGG vector backbone. (D) VP64-fusion plasmids, ubiquitously expressing MCP-VP64 and dCas9-VP64 activating fusions.

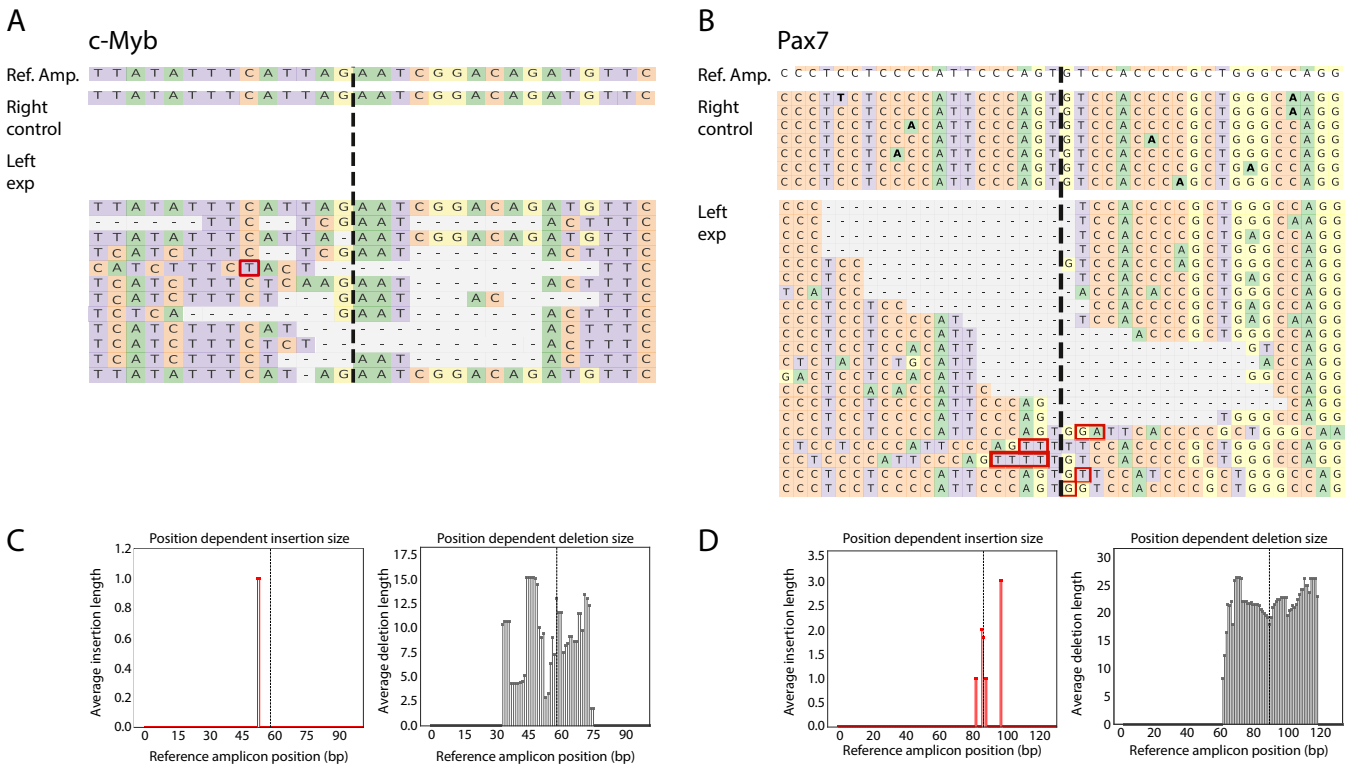


Figure S2. NGS validation of genome editing events produced by U6.3-driven sgRNAs against c-Myb and Pax7 with ubiquitous Cas9. (A, B) Alignments of a subset of insertion/deletion variants identified in experimental embryos treated with sgRNAs to c-Myb and Pax7 respectively. Indels predominantly occur close to the Cas9 cleavage site indicated by dotted line. SNPs in the control seqeunces are higlighted in bold. (C, D) Sashimi plots of the subset of reads filtered specifically for insertions and deletions within the targeted regions and analysed amplicons show size and relative position of the indels for each c-Myb and Pax7 respectively.

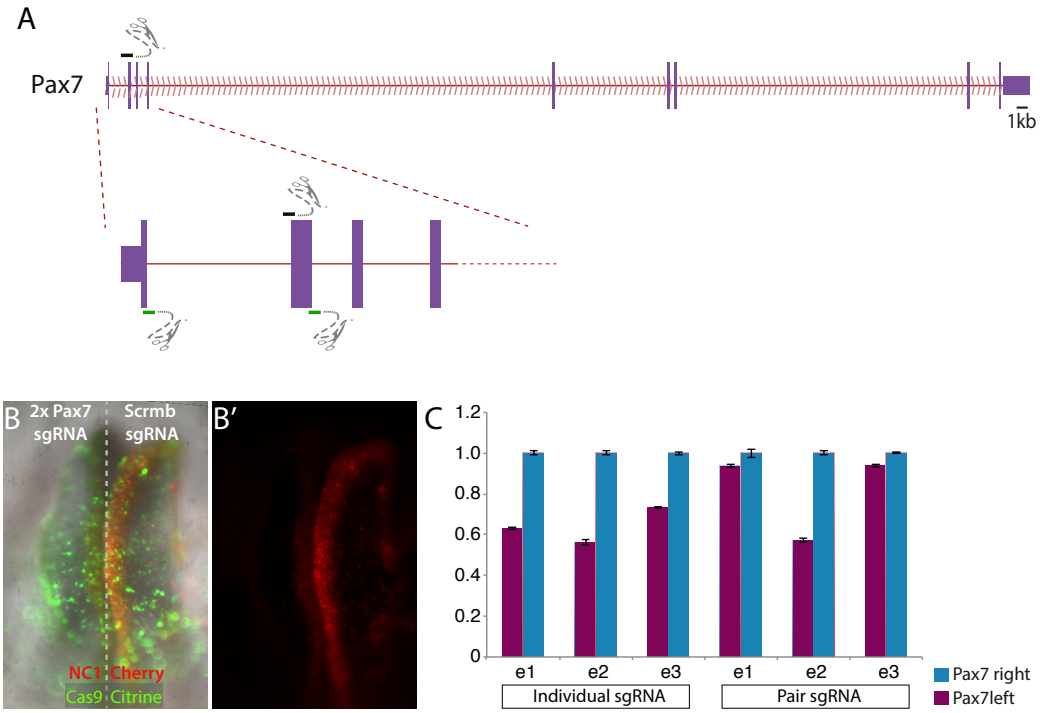


Figure S3. Using multiple sgRNAs does not increase the effect of GE-mediated upstream TF knockout on enhancer activity. (A) Schematic of the genomic locus of *Pax7* gene showing sgRNA location. Two sgRNAs selected at the splice donor sites flanking the essential 2nd exon of the *Pax7* gene are indicated in green and the position of the single sgRNA targeting the splice acceptor site of the same exon is shown in black. (B, B′) GE mediated disruption of Pax7 using two sgRNAs results in a decrease in NC1 driven mCherry on the experimental, left side of the embryo. (C) *Pax7* expression is down-regulated on the experimental (left magenta) versus the control (right, blue) side of the embryo following GE targeting with one or two sgRNAs.

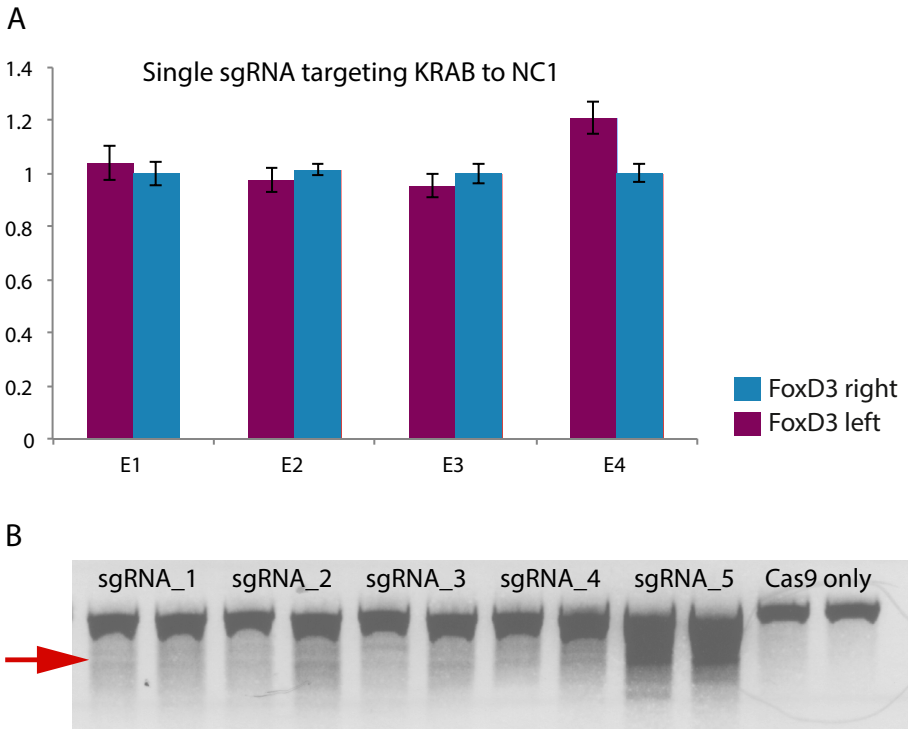


Figure S4. Targeting dCas9-KRAB with a single sgRNA to NC1 does not effect *FoxD3* expression. (A) A single sgRNA targeted to NC1 enhancer, co-electroporated with dCas9-KRAB has no effect on endogenous *FoxD3* expression on the experimental versus control side (n=12 from three individual experiments, representative embryos are shown). (B) sgRNAs are screened for activity using the T7-endonuclease assay. Embryos electroporated with sgRNA+Cas9 show multiple bands (red arrow) following T7 digestion when compared to Cas9 controls.

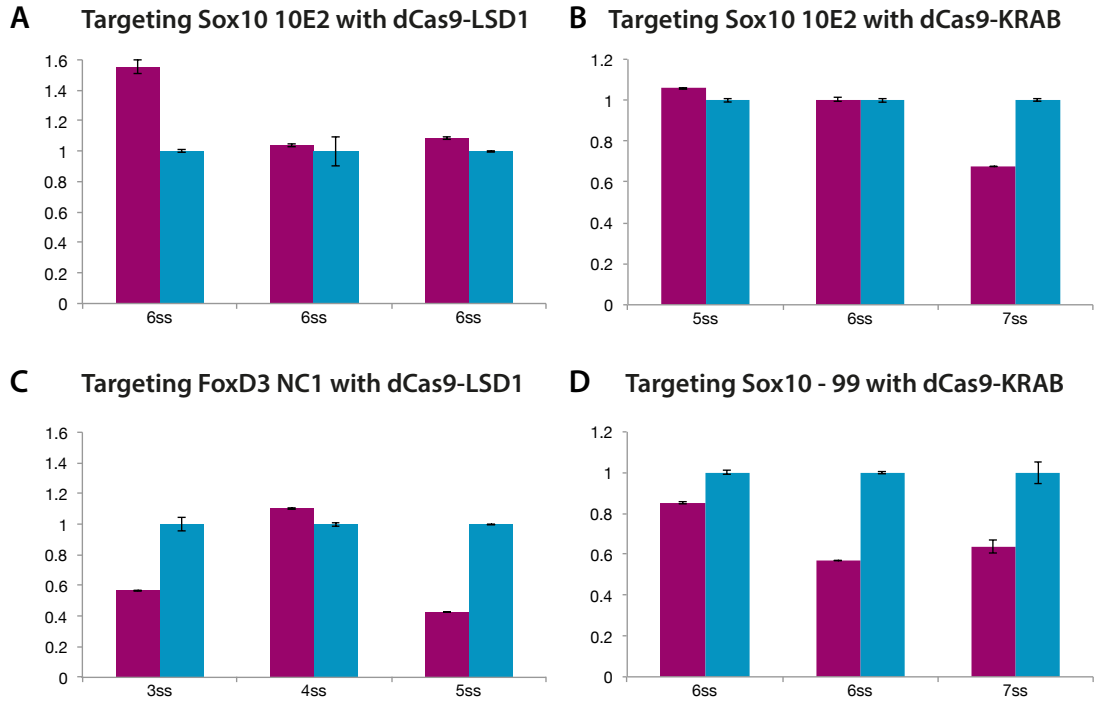


Figure S5. Epigenome engineering at targeted enhancers. (A) Targeting the Sox10 enhancer 10E2 with dCas9-LSD1 resulted in no significant change in Sox10 expression on experimental side (left, magenta) compared to control side (right, blue). (B) Targeting the Sox10 enhancer 10E2 with dCas9-KRAB causes a moderate reduction of Sox10 expression (33.3% of embryos assayed). (C) Targeting dCas9-LSD1 to the FoxD3 enhancer NC1 causes a moderate reduction of FoxD3 expression on the experimental compared to control side (44% of embryos assayed). (D) Targeting the Sox10 enh-99 with dCas9-KRAB causes a reduction in Sox10 expression on the experimental compared to control side (62.5% of embryos assayed). n>10 in all experiments. Results confirmed in three independent experiments; representative embryos are shown, error bars represent the standard deviation.

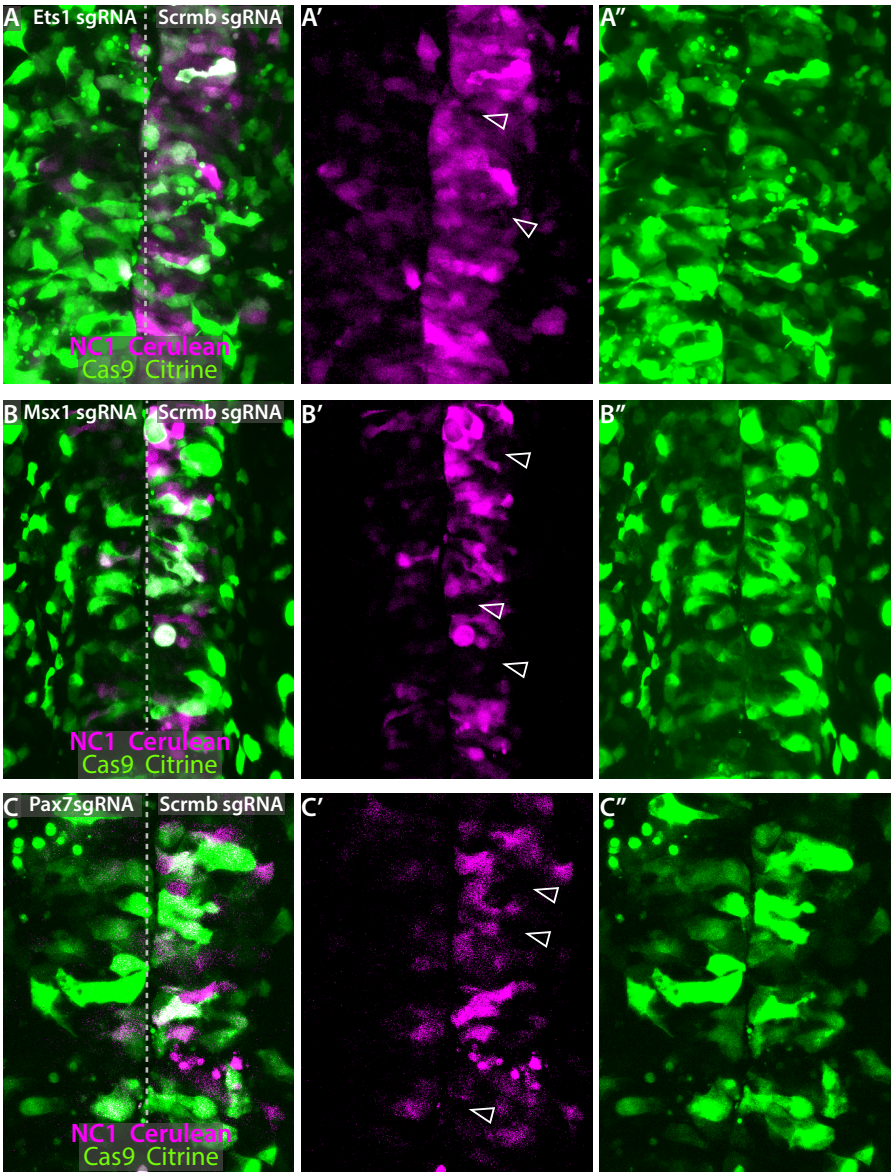


Figure S6. Targeting upstream transcription factors reduces NC1 driven Cerulean expression.
(A, B, C) Target sgRNA, Cas9-2A-Citrine and NC1-Cerulean constructs are co-electroporated on the left and scrambled control sgRNA with the same components on the right side of the embryo. GE-mediated disruption of Ets1 (A, A′, A″), Msx1 (B, B′, B″) and Pax7 (C, C′, C″) results in a decrease in NC1 driven Cerulean on the experimental, left side of the embryo (Cerulean shown in magenta). (A´, B´, C′) NC1-Cerulean alone clearly shows reduction in NC1 driven Cerulean expression on the experimental (left) side of the embryo. (A″, B″, C″) Cas9-2A-Citrine driven by the ubiquitous CAG promoter. Arrowheads indicate Cerulean-negative cells.

sgRNA cloning into chick U6.3 Mini Vector

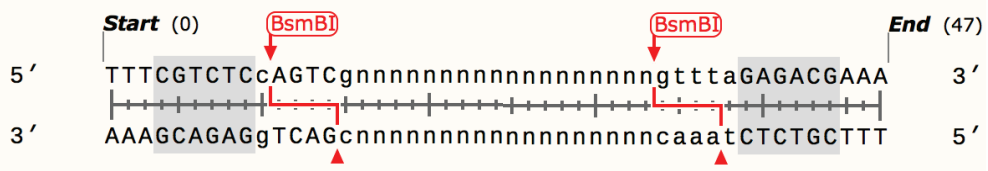
Materials

- Custom ordered forward and reverse oligos containing spacer RNA target sequence
- $ST_{low}E$ (50mM NaCl, 10mM Tris pH8.0, 0.1mM EDTA)
- pcU6.3 sgRNA Mini Vector stock (75ng/µl)
- BsmBI Type IIS restriction enzyme (NEB R0580)
- T4 DNA Ligase (NEB M0202)

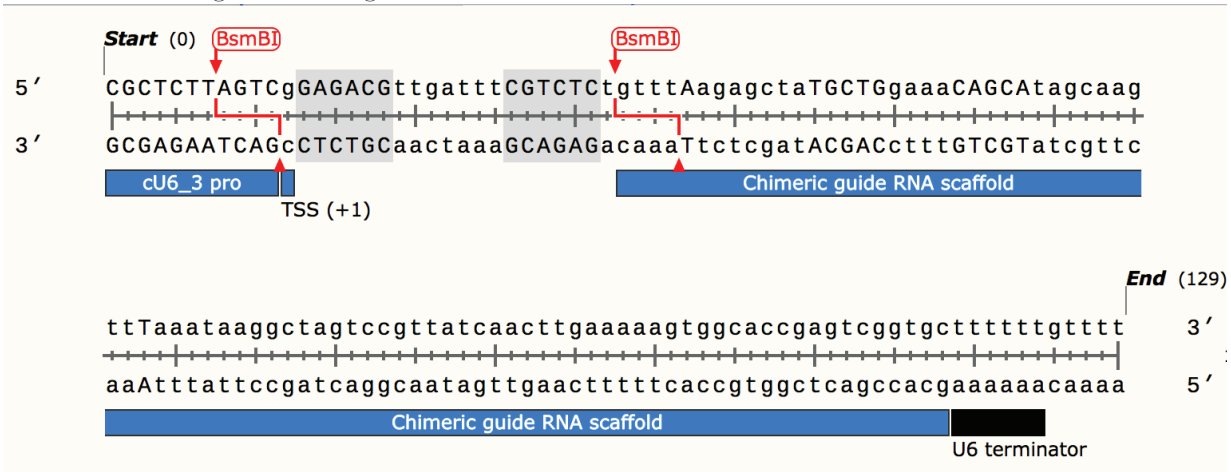
spacer RNA design

Identify potential Cas9 target sites (18-20 nucleotides) by scanning the genomic region of interest for PAM sequences (NGG) manually. Survey identified sequences genome-wide (BLAT) to ensure that the selected sequence has only a single hit in the genome and run through oligo-calc to identify any potential self annealing sequences. Order forward and reverse oligos for each guide recognition sequence (18-20nt) directly preceding the PAM (NGG). Do not include the PAM. If possible the sequence should include a G base at the 5' end, as this is required for polIII to initiate transcription from a U6 promoter. If such sequence cannot be selected a G residue should be added upstream of the selected recognition sequence. TracrRNA portion of the guide is included in the U6.3 mini expression vector. The oligo design is indicated in the diagram below.

Guide RNA Oligo Design:



U6.3 Mini Vector gRNA cloning site:



spacer RNA template preparation

1. Re-suspend oligos in $ST_{low}E$ at 100μM

2. Setup the annealing reaction as follows:

volume (μl)	reagent
0.5	FWD Oligo 100μM
0.5	REV Oligo 100μM
49.0	$ST_{low}E$

3. Anneal oligos using the following program on the PCR machine:

Temperature (°C)	Time (minutes)
94	5
64	temperature declining at 1°C/minute for 30 minutes

4. Run 5 μl of reaction on a 2% gel to confirm correct size

5. Nanodrop

Modified GoldenGate Assembly

1. Set up the reaction as follows:

volume (μl)	reagent
x	75ng pcU6.3 vector
x	4ng annealed oligos
2.0	10X T4 Ligase buffer
1.0	BsmBI NEB R0580
1.0	T4 DNA ligase NEB M0202
x	Water to final volume 20μl

2. Run the following programme in a PCR machine:

Temperature (°C)	Time (minutes)	No. cycles
37	5	
16	10	10
50	10	
80	10	
4	hold	

3. Samples can be directly transformed/cloned. The vector is tetracycline resistant. We recommend performing a negative control reaction with no oligos, to ensure no un-cut plasmid contamination. Preps can be sequenced with U6-FWD primer gatcaagcctgattgggagaa

HRMA

Materials

- Purelink genomic DNA extraction mini kit (Life Technologies K1820-02)
- C1000 TouchTM Bio-Rad Thermal Cycler or similar
- LC Green plus Dye (BioFire Diagnostics, BCHM-ASY-0005)
- Diamond Master Mix (Client life science- Cat no: HS002-TS)
- Custom designed Forward and Reverse primers 10µM
- TlowE (50mM NaCl, 10mM Tris pH8.0, 0.1mM EDTA)
- 384-well hard-shell PCR plates (Biorad No.hsp3805r)
- MicroAmp Optical Adhesive Film (Life Tech 4311971)

Instructions

1. Isolate genomic DNA from single embryos using Purelink genomic DNA mini kit. Collect at least 4 biological replicates (4 individual embryos)

2. Resuspend HRMA primers (100-150bp target region) in TlowE to 100µM and dilute in ddH_20 to get 10µM working stock

3. Prepare the HRMA reaction mix, as below, make a master mix as appropriate for a number of samples necessary (NB: LC Green/LightCycler R 480 High Resolution Melting Master Mix are light sensitive so keep covered with foil)

volume (µl)	reagent
1.0	10x LC Green plus Dye (BioFire Diagnostics, BCHM-ASY-0005)
5.0	2x Diamond Master Mix (Client life science- Cat no: HS002-TS
0.5	Fwd Primer 10µM
0.5	Rev Primer 10µM
x	gDNA (10-100ng)
x	water up to 10µl final volume

4. Add 10ul of the HRMA reaction mix to each well being used of a HRMA plate (384-well hard-shell PCR plates- Biorad No.hsp3805r)

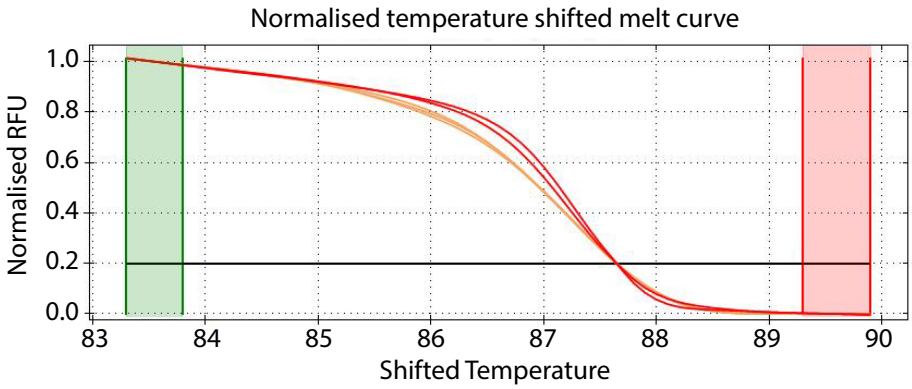
5. Secure the plate with clear film (microAmp optical adhesive film Life Tech 4311971)

6. Pulse centrifuge to maximum speed to ensure all liquid is at the bottom of the well and to minimise bubbles

7. Run the following programme on the C1000 TouchTM BioRad Thermal Cycler

Temperature (°C)	Time (seconds)	No. cycles
95	5 minutes	
95 60 72	20 20 30	49
72	2 minutes	
95	30	
25	30	
95	30	
Melt curve 65-95 increment 0.2 for 0.02 seconds		

8. When finished, save and open BioRad Precision Melt analysis. Generate and compare normalised temperature-shifted melt curves for control (Cas9 only) and experimental (Cas9 + sgRNA). If the sgRNA is cutting effectively the shift will be clear as shown in the example below, where Cas9 only controls are shown in red and experimental sgRNA samples are shown in orange.



T7 Endonuclease Assay

Materials

- Purelink genomic DNA extraction mini kit (Life Technologies K1820-02)
- Promega Wizard SV gel extraction kit (A9282)
- 0.25M EDTA
- T7 endonuclease I (NEB M0302L)
- Custom designed Forward and Reverse primers 10μM

Instructions

1. Isolate genomic DNA from single embryos using Purelink genomic DNA mini kit. Collect at least 4 biological replicates (4 individual embryos)

2. Amplify region of interest, ~1kb, using a high fidelity proof reading enzyme of your choice. Gel purify target region, elute in water

3. Add 2μl NEB buffer 2 to ~200 ng of purified PCR product and dH_2O to a total of 19μl in a PCR tube

4. Run hybridisation reaction in a PCR cycler:

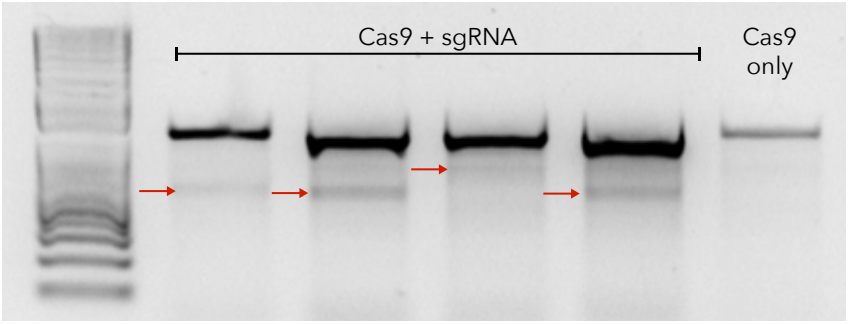
95 °C 5 mins
Ramp down to 85 °C at 2 °C/s
Ramp down to 25 °C at 0.1 °C/s
Hold at 4 °C

5. Add 1μl (10U) T7 endonuclease I and incubate at 37 °C for 15 mins

6. Stop the reaction by adding 2μl of 0.25M EDTA

7. Run on a 1.5% agarose gel

8. Compare control (Cas9 only) and experimental (Cas9 + sgRNA) samples. If the sgRNA is cutting effectively extra band(s) will be observed when compared to control, as shown in the example below.



NGS validation of GE events

Materials

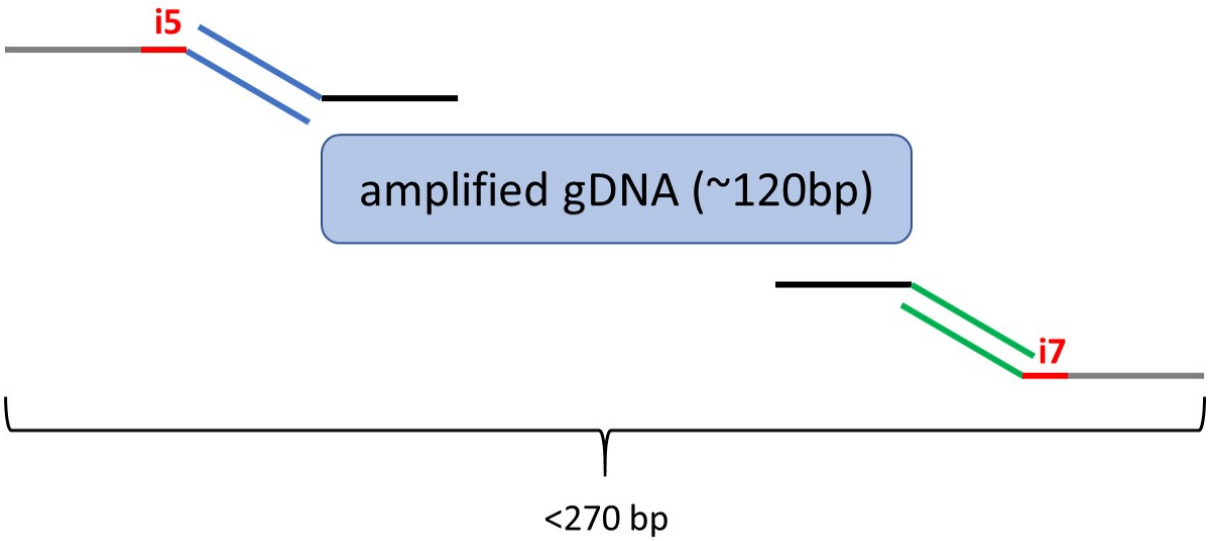
- Phusion High-Fidelity DNA Polymerase (NEB M0531L)
- Wizard SV gel extraction kit (Promega A9282)
- Nextera XT Index kit (Illumina FC-131-1001)
- Tapestation D1000 (Agilent 5067-5582)
- Qubit dsDNA HS Assay kit (Thermo Fisher Scientific Q32854)
- KAPA library quantification kit for NGS (KAPA Biosystems/Roche KK4835)
- MiSeq 300 cycles reagent kit (Illumina MS-102-2002)

Primer design

1. If using the 300 cycles sequencing kit, keep the final size of the library <270bp so that the sequencing reads from both ends will give a good overlap across the Cas9 cut site.

2. Design primers that will amplify a ~120bp region of gDNA spanning across the predicted Cas9 cleavage site and attach the tails listed below.

F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[gene-specific primer]
R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[gene-specific primer]



Library preparation (1st PCR)

1. Dilute the gDNA to 5ng/μl and set up 5 individual reactions per sample as follows:
   25 μl Phusion
   2.5 μl F primer (10 μM)
   2.5 μl R primer (10 μM)
   19 μl water
   1 μl gDNA

2. Run PCR as below:
   98°C 30 sec
   5 cycles:
      98°C 10 sec
      57°C 30 sec
      72°C 30 sec
   25 cycles:
      98°C 10 sec
      66°C 30 sec
      72°C 30 sec
   72°C 10 min

4. Run 5 μl on an agarose gel; if the correct size is confirmed, PCR purify the remaining 45μl and dilute 1:100 in water.

5. For each sample, make a pool with the 5 purified and diluted individual reactions (to be used as template in the second PCR).

Library preparation (2nd PCR)

1. Set up as follows:
   25 μl Phusion
   5 μl N ### primer (i7)
   5 μl S ### primer (i5)
   10 μl water
   5 μl pool from 1st PCR

2. Run PCR as below:
   98°C 30 sec
   15 cycles:
      98°C 10 sec
      66°C 30 sec
      72°C 30 sec
   72°C 10 min

3. Run the reaction on an agarose gel, cut the band of the correct size and gel purify.

Library quantification and sequencing

1. Quantify each library by Qubit and check the size on Tapestation.

2. Pool the libraries for sequencing and quantify the pool by KAPA.

3. Set up the MiSeq run.

Data analysis

1.After trimming, merge the forward and reverse reads using FLASH (Fast Length Adjustment of SHort reads).

2. Create an artificial chromosome with the sequence of the amplified gDNA.

3. Use bowtie2 to map the merged reads to the artificial chromosome.

4. Visualise the data on IGV / Quantify the NHEJ events with CRISPResso.

Table 1.

Optimized guide RNAs

Target	Sequence
FoxD3	GAAGAAGCTGACGCTCAG
Ets1	TTGGATGGGTCCACTGCC
Msx1	GAGGAGAGGAAGAGACAC
Pax7	AGTGTCCACCCCGCTGGGCC
c-Myb	TTTTATATTTCATTAGAAT
Sox9	CTCTCATTCAGCAGCCTG
NC1 sgRNA.2	GCAGCATGGATAACATCC
NC1 sgRNA.3	GTGTATGTAAATGTGCAT
NC1 sgRNA.4	GAAGCTCATTAGATATTCCC
NC1 sgRNA.5	CTAAGTAATTAGATTTTAAC
NC1 sgRNA.6	TAAAAGAGAATTTCTTTCAG
10E2 sgRNA.1	AGCAGGAGCAGGGAAACAAT
10E2 sgRNA.2	AAACATAAGCACAAACTAGG
10E2 sgRNA.3	TGGTAAGGATGGCCTGGATC
10E2 sgRNA.4	GCTGGGGAGGGGAGGCGGGC
10E2 sgRNA.5	CCATATCAACCATTCTCCAG
99 sgRNA.1	GGTGAGAAATGTTGAAAACG
99 sgRNA.2	GTGTGTGACTCTTTTGTTCC
99 sgRNA.3	TGGACAACTTTGCTAGGCCC
99 sgRNA.4	GCACAAAGGAAATGGATAAA
99 sgRNA.5	AGGAAAGAGGGAAGGAAGGC
Sox10 promoter	CAAGAGGCATCCAGCCGGG
Scrambled	TGCAGTGCTTCAGCCGCT