

Protocol for tissue-specific gene disruption in zebrafish

Overview

This protocol describes a method to inactivate genes in zebrafish in a tissue-specific manner. It can be used to analyze mosaic loss-of-function phenotypes in F0 embryos or to generate stable tissue-specific knock-out lines. It takes advantage of the Tol2 transposase technology to integrate in the fish genome a vector expressing a guide RNA (gRNA) from a ubiquitous zebrafish U6 promoter and Cas9 under the control of a tissue-specific promoter. This protocol comprises 5 steps: 1) the identification of efficient CRISPR target sequences in the gene of interest; 2) the annealing of gene-specific oligonucleotides; 3) the construction of the tissue-specific CRISPR vector; 4) the injection of the CRISPR construct in zebrafish embryos; 5) the phenotypic analysis and generation of stable lines (Fig. P1).

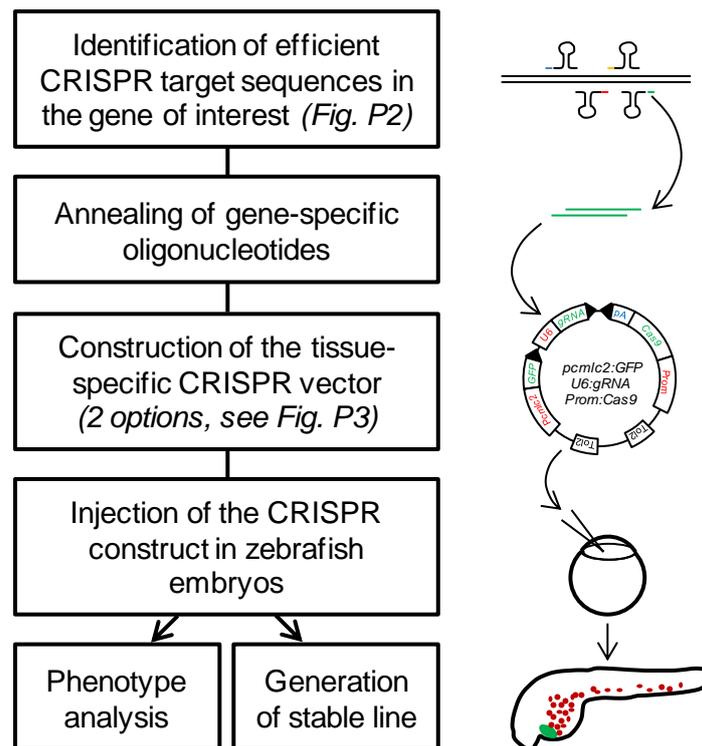


Figure P1 – Workflow of tissue-specific gene disruption

Reagents

- Destination vector for Gateway (e.g. pDestTol2 cmlc2:GFP, U6:gRNA or pDestTol2pA2 U6:gRNA, available through Addgene)
- Middle entry vector for Gateway containing Cas9 (e.g. pME-Cas9 or pME-Cas9-T2A-GFP, available through Addgene)
- 5' entry vector for Gateway containing the tissue-specific promoter of interest
- 3' entry vector for Gateway containing a polyA sequence (p3E-polyA, vector #302 from the Tol2Kit)
- Gene-specific oligonucleotides (see below)
- BseRI enzyme (New England Biolabs)
- Gateway LR clonase II (Invitrogen)
- T4 DNA ligase (New England Biolabs)
- Gel extraction kit or PCR-purification kit (Qiagen)

Procedure

1. Identification of efficient CRISPR target sequences in the gene(s) of interest

Pick several (3 to 6) CRISPR target sequences in each gene of interest using available tools (Hsu et al., 2013; Montague et al., 2014) (Fig. P2). Produce the gRNAs *in vitro* as per usual procedures (Gagnon et al., 2014; Hwang et al., 2013). Inject the gRNAs along with Cas9 protein or mRNA into one-cell stage embryos of a WT strain. Extract DNA from injected embryos at 24 or 48 hpf by the HotSHOT method (Meeker et al., 2007) and assess mutation rates at target loci by sequencing or enzymatic assays (e.g. T7E1 assay, Surveyor assay) (Gagnon et al., 2014; Kim et al., 2009). Proceed with the target sequences that have shown effective targeting (we usually only keep target sequences for which the mutation rates exceed 10%).

Note: *alternatively, it is possible to test the efficiency of target sequences directly in the context of the Tol2 vector by cloning various target sequences in the U6:gRNA cassette of a vector expressing Cas9 under the control of a ubiquitous promoter.*

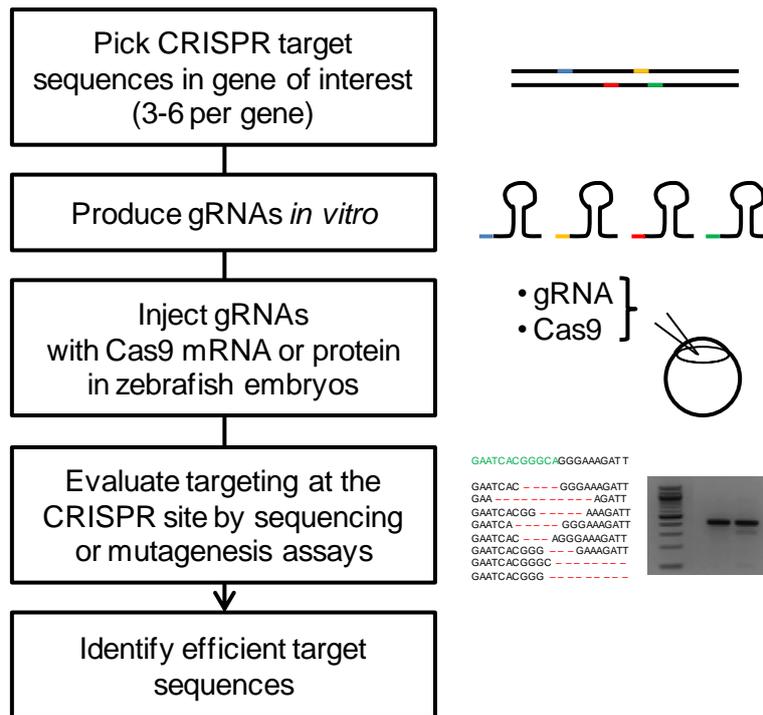


Figure P2 – Identification of efficient CRISPR target sequences

2. Annealing of gene-specific oligonucleotides

Order 22-mer, unmodified oligonucleotides as follows:

Forward: target sequence (20 bases)-GT

Reverse: reverse complement of target sequence-GA

Note: the target sequence must start with a G. If not, replace the first base by a G. This will introduce a mismatch but most mismatches at the 5' end of the target sequence are well tolerated.

Example: for CRISPR target sequence GGTGGGAGAGTGGATGGCTG, order GGTGGGAGAGTGGATGGCTGGT (forward) and CAGCCATCCACTCTCCCACCGA (reverse).

Anneal the two oligos in thermocycler (5 min. at 95°C, -1°C/min. down to 20°C).

3. Construction of the tissue-specific CRISPR vector

There are two options to create a tissue-specific CRISPR vector. The order in which you perform the cloning steps depends on the option you choose.

Option 1: Clone the gene-specific seed sequence into the destination vector and then perform the Gateway reaction with the tissue-specific promoter and Cas9 (Fig. P3).

This option will be used preferentially to inactivate the same gene or a few genes in several different tissues (e.g. using various tissue-specific promoters with few different gRNAs) or if the tissue-specific promoter of interest contains one or several BseRI restriction sites.

Option 2: Perform the Gateway reaction first to assemble a vector for tissue-specific expression of Cas9 and then clone the gene-specific seed sequence into the U6:gRNA cassette (Fig. P4).

This option is better suited for targeting many different genes in the same tissue (e.g. using a single tissue-specific promoter with many different gRNAs). If you wish to use option 2 with a tissue-specific promoter containing one or several BseRI sites, you may consider modifying them by site-directed mutagenesis on the 5' entry vector for Gateway.

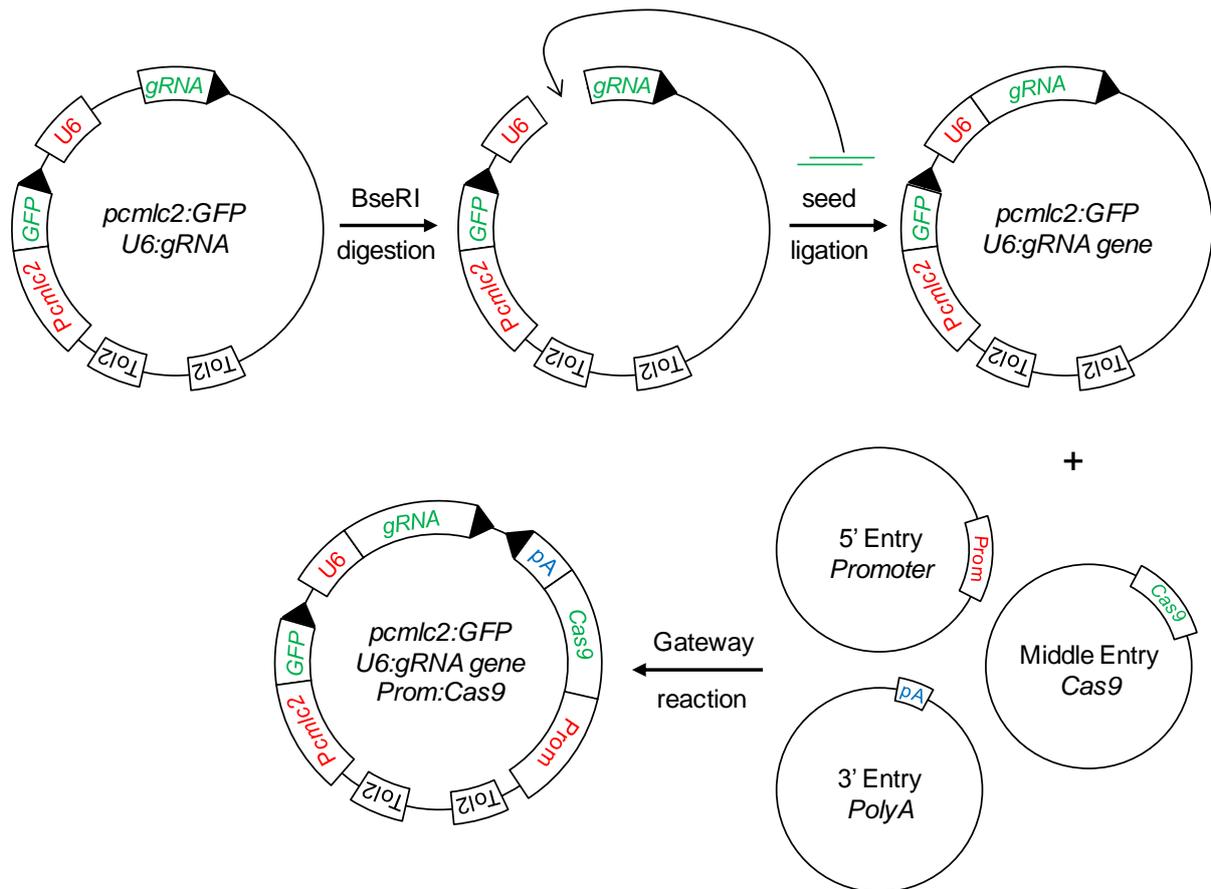


Figure P3 – Construction of a tissue-specific CRISPR vector, option 1

Option 1

- Digest the destination vector of your choice with BseRI enzyme.
- Purify on 1% agarose gel or column.
- Ligate the gene-specific seed sequence into BseRI-digested destination vector (1:20 vector:insert molar ratio).
- Transform chemically competent *ccdB*-survival bacteria, plate on LB Agar and select with ampicillin + chloramphenicol.
- Extract plasmid DNA from 2-3 distinct colonies and sequence using the following primer: CCTCACACAAACTCTGGATT.
- Perform the Gateway reaction (Hartley et al., 2000) with the destination vector, a 5' entry vector containing a tissue-specific promoter of interest, a middle entry vector containing zebrafish codon-optimized Cas9 and p3E-polyA, according to manufacturer's protocol.
- Transform chemically competent bacteria, plate on LB Agar and select with ampicillin.
- Extract plasmid DNA from 2-3 colonies and check correct recombination by digestion or sequencing.

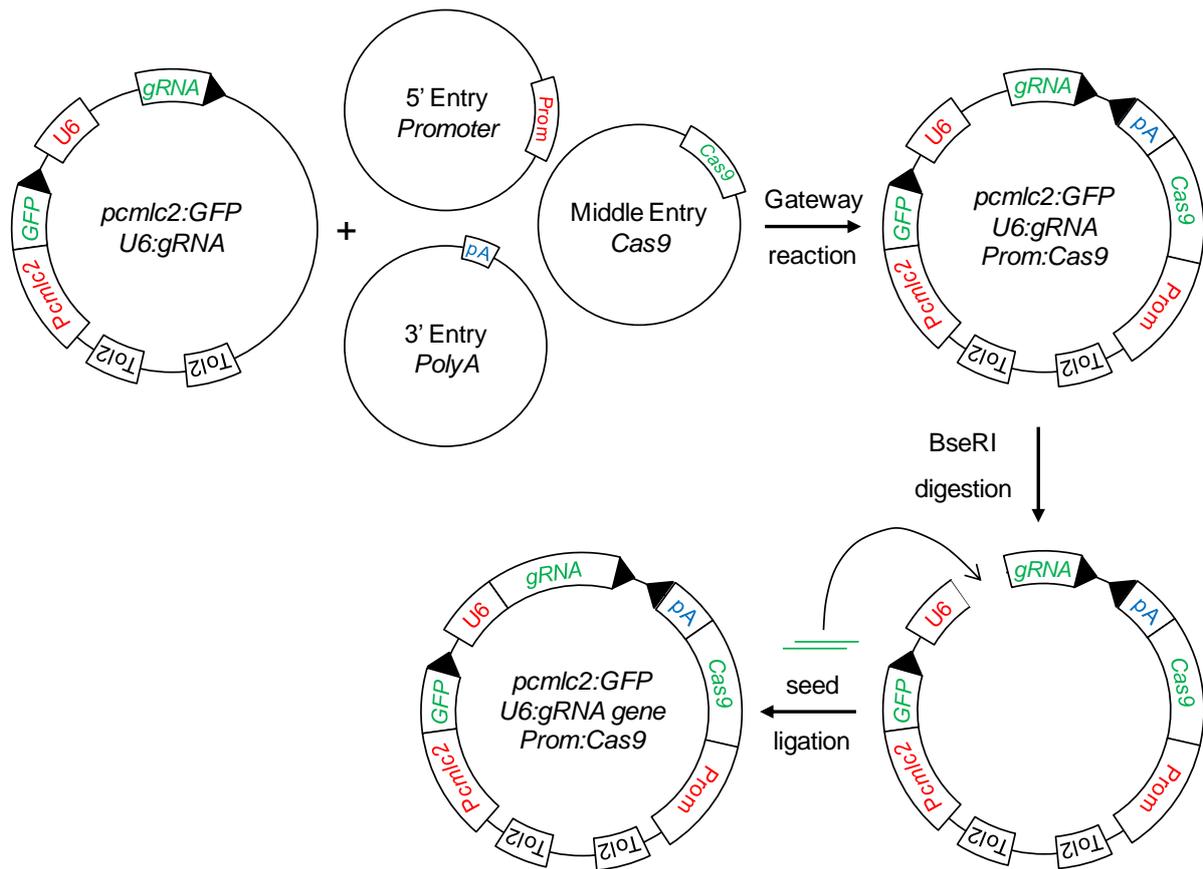


Figure P4 – Construction of a tissue-specific CRISPR vector, option 2

Option 2

- Perform the Gateway reaction (Hartley et al., 2000) with the destination vector of your choice, a 5' entry vector containing a tissue-specific promoter of interest, a middle entry vector containing zebrafish codon-optimized Cas9 and p3E-polyA, according to manufacturer's protocol.
- Transform chemically competent bacteria, plate on LB Agar and select with ampicillin.
- Extract plasmid DNA from 2-3 colonies and check correct recombination by digestion or sequencing.
- Digest the resulting CRISPR vector with BseRI enzyme.
- Purify on 1% agarose gel or column.
- Ligate the gene-specific seed sequence into BseRI-digested destination vector (1:20 vector:insert molar ratio).
- Transform chemically competent bacteria, plate on LB Agar and select with ampicillin.
- Extract plasmid DNA from 2-3 distinct colonies and sequence using the following primer: CCTCACACAACTCTGGATT.

4. Injection of the CRISPR construct in zebrafish embryos

Mix and inject 20-30 pg of CRISPR vector and 20 pg of Tol2 mRNA into one-cell stage embryos (Kawakami et al., 2004). For reliable phenotypic analyses, we recommend injecting >50 embryos per construct. Vectors expressing gRNAs targeting an irrelevant gene or driving Cas9 expression in a different tissue can be used as negative controls.

Allow injected embryos to develop at 28.5°C.

5. Phenotypic analysis and generation of stable lines

Sort injected F0 embryos based on the expression of a transgenesis marker present either in the destination vector (e.g. *cmlc2*:GFP) or in the middle entry part of the vector (e.g. T2A-GFP). Only positive embryos should be considered for further analysis.

For the generation of stable lines, raise positive F0 fish to adulthood. Back-cross them to the strain used for injection and sort positive F1 embryos (according to the expression of the transgenesis marker). F1 embryos can be analyzed phenotypically or raised to adulthood.

Note: depending on the transgenesis marker used, it may be possible to evaluate the level of mosaicism in injected embryos. In that case, injected embryos could be further sorted according to their level of mosaicism.

References

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