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Zebrafish enhancer reporter assay

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Seppe De Winter[1], Valérie ercier[1], Katina Spanier[1]

[1]Lab of Computational Biology

Seppe De Winter
Lab of Computational Biology

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Protocol status: Working
We use this protocol and it's working

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Abstract

Protocol describing enhancer reporter assay in zebrafish embryos. Including enhancer reporter cloning, zebrafish microinjection and imaging.

Order suitable enhancer reporter plasmid

1 Considerations:

- This protocol makes use of Tol2-transposase system to integrate the enhancer-reporter construct in the zebrafish's genome: make sure that the vector has **Tol2 sites**.
- It is useful to have a control for successful injection/integration events. For this purpose a vector containing a constitutive reporter that is cell type-specific and that drives another fluorescent protein as the enhancer reporter can be used.
- Promoter of the enhancer reporter should not be too strong. The amount of expression from the enhancer-reporter gene should be minimal when the enhancer is not active (or not present in the vector).

Example plasmid: **Addgene 194518**
✅ This plasmid can be used for Tol2 based integration
✅ This plasmid drives constitutive mCherry expression in the zebrafish's eye.
✅ This plasmid uses the ZSP promoter, which shows minimal activity without an enhancer.
✅ The enhancer drives EGFP expression.

Order enhancers

2 Order enhancer sequences with either constant flanking sequences **or** sequences that are homologous to the target plasmid. In the first case an extra step of PCR has to be performed in order to introduce the homologous sequences at the enhancer flanks. The homologous sequences should be designed in such a way that the enhancer will be cloned in the vicinity of the minimal promoter and the reporter gene. If the target plasmid is linearised using restriction digestion (as is described here), the restriction target sites should also be taken into account.

For example, for **Addgene 194518** following sequences can be used:

```
5’ TTAGGGATAACAGGGTAATCGCGAATTGGGTACCGGGC 3’
5’ CTTTCAACAAGCCCGAAAGATCTTCTGGAAGCCTCCAGTGAATT 3’
```

Later, resulting in the following integration:

```
5' ... TTAGGGATAACAGGGTAATCGCGAATTGGGTACCGGGC [ENHANCER]
AATTCACTGGAGGCTTC CAGAAGATCTTTCGGGCTTGTTGAAAG ... [ZSP] [EGFP] ...
3'
```

Optional: PCR amplify enhancer to introduce homologous sequence

3 Design and order suitable primers. For example, if the enhancers have the following constant flanking sequences:

```
5' ATATACCCTCTAGAGTCGAA 3'
5' GATTACCCTGTTATCCCTAA 3'
```

Following primers can be used:

```
5' ttagggataacagggtaatcATATACCCTCTAGAGTCGAA 3'
5' ctttcaacaagcccgaaagatcTTAGGGATAACAGGGTAATC 3'
```

Where the non-capitalised nucleotides are homologous to the plasmid

4 Prepare PCR reaction

- Forward primer: [M] 300 nanomolar (nM)
- Reverse primer: [M] 300 nanomolar (nM)
- Template: [M] 0.1 ng/ul
- KAPA HiFI HotStart ReadyMix 1x

5 Perform PCR

1 cycle
- 3 minutes 95C

20 cycles
- 20 seconds 98C

- 15 seconds 65C
- 15 seconds 72C

1 cycle
- 2 minutes 72 C

6 PCR clean up

Follow: PCR clean up protocol (e.g., **NucleoSpin Gel and PCR Clean-up**)

Clone enhancers in reporter plasmid

3h 35m

7 Linearise plasmid using PCR or restriction digest.

For **Addgene 194518** and the adaptor sequences mentioned above linearisation can be performed using:

```
BglII and XhoI
```

7.1 Create reaction mix:
- rCutSmart buffer (1x)
- 7 µg plasmid
- 10 U BglII
- 20 U XhoI
- Add H2O to total volume of 20 µL

7.2 Incubate 02:00:00 at 37 °C

2h

8 Clean up

Follow: PCR clean up protocol (e.g., **NucleoSpin Gel and PCR Clean-up**)

9 Perform NEBuilder reaction with linearised plasmid and enhancer fragments

9.1 Create reaction mix: 45m

- Linearised plasmid and insert at an 1:2 ratio

  Use following formula $50ng \cdot \frac{2 \cdot length_{insert}}{lenght_{plasmid}}^{-1}$

- 1x enzyme mix

Incubate at 50 °C for 00:45:00

9.2 thaw Stellar chemically competent bacteria on ice for 00:15:00 15m

9.3 Add 2.5 µL of reaction mix to 20 µL of bacteria

9.4 Keep 00:30:00 on ice 30m

9.5 45 second heat shock at 42 °C

9.6 Keep 00:05:00 on ice 5m

9.7 Plate on on Carbenicillin plates and incubate at 37 °C Overnight

9.8 Pick single colonies and incubate Overnight in 10 mL LB medium with Carbenicillin at 37 °C

10 Isolate plasmids using **QIAprep Spin Miniprep kit** and elute twice for a total of 50 µL

11 Sequence plasmid prep using Sanger sequencing

For the plasmid **Addgene 194518**, the following primer can be used:

```
5'-CGACTCACTATAGGGCGAATTGGG-3'
```

Tol2 Transposase mRNA synthesis

2h 35m

12 Order suitable plasmid for *in vitro* Tol2 Transposase expression (see Kwan *et al.*, https://doi.org/10.1002/dvdy.21343). For example, **pCS2FA**

13 Linearize plasmid. pCS2FA can be linearized using NotI-HF.

13.1 Prepare reaction mix:
- rCutSmart Buffer (1x)
- 5 µg plasmid
- 20 U NotI-HF
- Add H2O to total volume of 50 µL

13.2 Incubate Overnight at 37 °C

13.3 Heat inactivate restriction enzyme at 65 °C fo 00:20:00 20m

14 Purify linearized plasmid using PureLink PCR cleanup kit (Invitrogen)

15 *In vitro* transcription of capped Tol2 mRNA with mMESSAGE mMACHINE™ SP6 Transcription Kit (Invitrogen)

15.1 Create reaction mix:
- SP6 reaction buffer (1x)
- NTP/CAP (1x)
- 1 µg linearized plasmid
- 2 µL SP6 RNA Polymerase Enzyme Mix
- Add H2O to a total volume of 20 µL

15.2 Incubate 02:00:00 at 37 °C 2h

15.3 Remove template DNA by adding 1 µL of TURBO DNase, mix and incubate 00:15:00 at 37 °C 15m

16 Purify Tol2 Transposase mRNA using ssDNA/RNA Clean & Concentrator Kit (Zymo research)

Collect zebrafish embryo's and inject enhancer construct 30m

17 Prepare Danieau's embryo medium (**100x**)

- NaCl [M] 1.74 Molarity (M)
- KCl [M] 21 millimolar (mM)
- MgSO4.7H2O [M] 12 millimolar (mM)
- Ca(NO3)2.4H2O [M] 18 millimolar (mM)
- HEPES [M] 150 millimolar (mM)

Bring to pH 7.6

18 Prepare agar plates

18.1 Boil [M] 2 Mass Percent agarose (Ultra Pure Invitrogen) in 50 mL Danieau's medium (1x)

18.2 Pour solution in 10cm petri dish

18.3 Place template plate (containing 6 slated rows; **World Precision Instruments**) on top of the agar (it will float) for 00:30:00 30m

18.4 Remove template plate and cover with Danieau's medium (1x) to prevent drying, store at 4 °C

19

20 Fabricate micropipettes by heading and pulling 11 mm borosilicate tubes (World Precision Instruments) using micropipette puller (Sutter-brown).

21 Prepare injection mix containing plasmid DNA ([M] 30 µg/µL) and Tol2 mRNA ([M] 40 ng/ul)

22 Prepare for injection

22.1 Turn on the Pneumatic microinjector system (PicoPump, World Precision Instruments, SUS-PV820) and set the injection pressure to 10-20 PSI and hold pressure to 0.2-0.5 PSI to prevent backflow

22.2 Backfill the needle with injection mix, using capillary action.

22.3 Check injection volume (2-4nl) by injecting a droplet in mineral oil on a micrometer slide. Based on droplet volume, adjust needle size (by breaking the tip) or adjust injection duration.

23 Collect 1 cell stage embryos and inject

23.1 Breeding is trigged by the light / dark cycle (zebrafish mate in the first few hours of each light cycle). Use breeding tanks with slotted bottoms to separate adults from the eggs. Collect the embryos at the **1-cell** stage and place them in Danieau's embryo medium (**1x**) with methylene blue on agar plate.

23.2 Use plastic pipettes to transfer the eggs to and from the injection plate

23.3 Arrange the eggs in the injection plate slits, with the cell pointing to the side

23.4 Remove Danieau's medium such that it's just covering the top of the agar (this prevents the eggs from floating)

23.5 Carefully push the needle through the chorion and position it into the cell at the one-cell stage. Press the pedal to delivery a droplet of working solution to the cell and remove the needle.

23.6 go to step #23.5 repeat for each egg

23.7 Transfer the eggs (carefully) to petridish containing Danieau's medium (1x)

23.8 Place petridish with eggs in incubator at 28.5 °C and raise for desired amount of time

Imaging

24 Select zebrafish with successful injection based on constitutive enhancer control (mCherry fluorescence in the eye in case of **Addgene 194518**) using widefield fluorescence microscope

25 Anesthetize fish using tricaine ([M] 0.02 Mass Percent)

26 Mount fish on fluorodish (FD3510-100. world precision instruments) in low melting agarose ([M] 1 Mass Percent)

27 Image fish on fluorescence microscope