

## Generation of transgenic zebrafish using Tol2

- Thomas S. Becker Lab, Pavla Navratilova -

### Transposase mRNA preparation

- digest template (pCS2-TP) with Not1 and extract with phenol-chlorophorm
- use mMessage Machine SP6 kit from Ambion to synthesize transposase mRNA (follow kit manual)
- purify according to manual using LiCl precipitation or better use MegaClear kit from Ambion
- aliquot mRNA by 5-10  $\mu$ l in 50-200 ng/ $\mu$ l concentration and store at  $-80^{\circ}\text{C}$ .

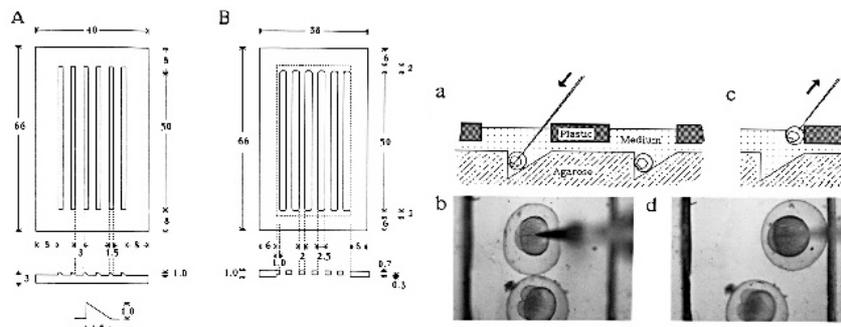
### Tol2 construct DNA preparation

- Miniprep ( 5 ml bacterial culture ) your Tol2 construct DNA and sequence ( high quality circular DNA will be needed for injection!)
- To remove RNAses phenol-chloroform extract and precipitate DNA and dilute to 50-200 ng/ $\mu$ l.
- (Use nuclease-free water, filter tips and gloves for all DNA/mRNA handling)

### Injection:

#### Injection chambers

- Pour approximately 20 ml of hot 1.5% agarose in water into a 100 x 15 Petri dish on a level surface. Wait until nearly solidified.
- Add an additional 20 ml of the 1.5% agarose to the dish. Set the plastic mold (teeth down) into the liquid agarose overlay, tapping to eliminate any bubbles.
- After the agarose hardens, remove the plastic mold and wrap the Petri dish in parafilm. This can be stored for several days at room temperature.



making needles:

- we use glass needles without filament id.0,58mm and Sutter Instruments machine with approx. settings:
- Heat: 675, Pull: 238, Vel: 145, Time: 120

prepare injection glass needles and fix them on the modeling clay strips into a petridish

#### Preparations the day before:

- the evening before injection keep males separate from females in mating boxes. Put them together the next morning, check the tank every ten minutes if eggs are layed and collect the eggs using a tea mesh. Rinse eggs with E3 solution and transfer them into a petridish
- prepare tubes with 5/10 $\mu$ l injection mixtures to fill the needles (25ng/ $\mu$ l DNA + 25ng/ $\mu$ l TP mRNA + Phenol red (2/4 $\mu$ l of 7mg/ml))

#### Injection procedure:

- load needle with 2  $\mu$ l of the mixture using Eppendorf loading tips. Cut the opening of the needle with sharp blade or forceps. It needs optimization with injector pressure – the aim is as thin as possible but able to go through chorion and giving the optimal drop volume
- line up one cell stage embryos (10'-30' p.f.) on the agarose platform. Inject about 200 embryos each construct
- inject about 2 nl into the cytoplasm. Injections into cytoplasm of very young stage is the most efficient. Try to inject as early as possible, when the cell just starts to rise. (injection under the cell into the yolk would require 5-10nl volume which results in lower survival).
- use remaining injection mixture to check on EtBr gel that Transposase RNA was intact
- collect the injected embryos gently with E3 using disposable plastic pipette (2ml) into a clean petri dish and incubate them (about 50 - 100 per dish) at 28°C for 6 hours.
- remove all unfertilized and dead eggs using a disposable plastic pipette. Avoid high density of eggs in dish and distribute the eggs until you have approximately 60 eggs per dish.

#### Day after injection:

##### I. Screening:

sort embryos under fluorescent microscope. From the 200 injected embryos take only the best 40, the ones that show most fluorescent expression. Choosing the best raises the chance that you select founder fish that have integrated the construct into the germline genome. Transfer the positive embryos into a new dish with E3/methylene blue (1 mg/ml - methylene blue prevents grow of fungus).

## II. Embryonic Excision Assay:

To ensure after injection that your insert has been excised from your Tol2 transposon DNA construct – and most probably also been introduced into the zebrafish genome.

10-24 hrs after injection pick at least 8 embryos and transfer them into PCR-strip tubes into 50µl TE buffer (pH 8) with PK (200ng/µl). Incubate at least 1hr at 55°C, occasionally shaking. After dissolving embryos, heat to 95°C for 10'.

This is the template for PCRs with Tol2eea primers.

for 25µl reaction: 2,5 µl BioTaq polymerase buffer

0,75 µl MgCl<sub>2</sub>

0,5 µl dNTPs

0,75 µl Tol2eeaF primer

0,75 µl Tol2eeaR primer

0,25 µl BioTaq polymerase

17,5 µl H<sub>2</sub>O

2 µl template

program: 95°C/2' – ( 95°C/15'' - 56°C/20'' - 72°C/15'' ) x 35 - 72°C/7'

Tol2eeaF: GGTGAGCCAGTGAGTTGATTGC

Tol2eeaR: CTTTCTGCTATGGAGGTCAGGTATG

If you see on the gel 480bp products in more than half of the lanes, raise the rest of embryos for screen, otherwise check your TP mRNA if is intact and inject again.

### Raising injected fish (founder fish, F0):

- Transfer 5 days old larvae into 250ml glass beaker into E3 with methylene blue. Remember to clean larvae every day!
- Hatchlings are fed twice a day with dry commercial start food (Novotom, JBL). On day 10 the fish shall start to eat Artemia (Silver Star, U.S.A.). Increase the feedings from twice a day to three times a day.
- The adult fish are kept in an isolated system with recirculating water at 28.5 C
- Feeding takes place three times a day. Watch your own fish!
- 2 month old fish should be at least 2.5 - 3 cm in length. If they are not – they didn't get enough food. Remember: between 1 and 3 month they need plenty of food.

### Breeding founder fish and screening F1 fish:

- Breed single founder fish with single wildtype fish
- collect eggs the next day and remove all unfertilized and dead eggs six hours later
- screen the embryos under fluorescent microscope and sort the fluorescent positives (different expression patterns go into different dishes and get different numbers/names)
- if you have enough positive embryos that show the same pattern (watch carefully!), transfer them into separate dishes

1. into a dish with E3 where you add 0.15% PTU (final PTU concentration: 0.003%). These embryos are for microscopic observations (photograph d1, d2, d3, d4, d5; also fix equivalent embryos in 4% paraformaldehyde and transfer to MeOH afterwards (4% Paraf, wash PBT, 50% PBT/MeOH, 3 x 100% MeOH – immunostain protocol-), store in MeOH at -20°C. Add your photographs immediately into the database – the database is a kind of lab journal)
2. into a dish with E3 including methylene blue. These embryos are for raising.

(to raise embryos that have never been exposed to PTU enhances the survival rate. If you don't have enough embryos, you can't fix them and need to raise the ones that you have photographed)

- Some elements can act as silencers, so in case where you don't have find any GFP positive, keep collecting by 50 embryos per founder and freeze those in 300 µl of extraction buffer. Once you have 8 tubes of negative pools, isolate DNA from them to screen also by PCR as follows:

### PCR screen

gDNA preparation from embryo:

- put the embryos in a 1.5 ml eppendorf tube;
- add 300µl Extraction buffer + 6ul protease K (stock by 10mg/ml) add before incubation)
- incubate 2 – 3 h at 56°C, vortex every 30 min;
- add 750µl (2,5 Vol.) 96% EtOH, mix well;
- centrifuge at 13,200 rpm, 10 min;
- remove supernatant and wash the pellet with 200µl 70% EtOH;
- centrifuge for 2 min, remove liquid and dry pellet;
- dissolve pellet in 100µl TE buffer for ~ 20 min at 56°C, vortex a few times;
- centrifuge 5 min at 13,200 rpm to clear the solution;

PCR:

1 x Master Mix		
10 x buffer		2.5µl
MgCl <sub>2</sub> (50mM)	0.75µl	
primer EGFPf		0.5µl
primer EGFPPr	0.5µl	
dNTP (10mM)		0.5µl
polymerase Taq (5U/µl)		0.25µl
H <sub>2</sub> O		19µl
<u>DNA</u>		<u>1µl</u>
		25µl

Program: 95/3' - 35x(95/50" - 58/50" – 72/1') - 72/7'

### Extraction buffer:

10mM Tris (pH 8,2)  
 10mM EDTA  
 200 mM NaCl  
 0,5 %SDS  
 200 µg/ml proteinaseK (add before incubation)

### Raising F1 fish

- feed and maintain like described above
- F1 fish from one tank can be mated in groups
- collect and sort embryos for documentation

Rule is: you need embryos from three founders that show the same pattern to evaluate the regulatory element that you've tested in your construct.

Once you are sure about the "right" expression pattern, kill the F1 lines (need to be documented before!!) that show enhancer traps or additional position effects. Killing decisions will be discussed on the project meetings. If three F1 lines express the same, keep only one and kill the other two.

### Calculating injection volumes

(use a 10  $\mu\text{m}$  micrometer, place a drop of mineral oil, inject into the mineral oil by varying the pressure)

volume of sphere = $\frac{4}{3}r^3$		
diameter of sphere (2r)	volume of sphere in $\mu^3$	volume of sphere in liters
1 $\mu$	0.5 $\mu^3$	
2 $\mu$	4.2 $\mu^3$	
5 $\mu$	65.4 $\mu^3$	
10 $\mu$	523.6 $\mu^3$	
20 $\mu$	4,188 $\mu^3$	=4.2 pl
25 $\mu$	8,181 $\mu^3$	=8.2 pl
30 $\mu$	14,137 $\mu^3$	=14.2 pl
40 $\mu$	33,510 $\mu^3$	=33.5 pl
50 $\mu$	65,450 $\mu^3$	=65.5 pl
75 $\mu$	220,893 $\mu^3$	=221 pl = 0.22 nl
100 $\mu$	523,599 $\mu^3$	=525 pl = 0.52 nl
125 $\mu$	1,025,000 $\mu^3$	=1025 pl = 1.03 nl
150 $\mu$	1,772,000 $\mu^3$	=1772 pl = 1.77 nl
175 $\mu$	2,814,000 $\mu^3$	=2814 pl = 2.81 nl
200 $\mu$	4,188,800 $\mu^3$	=4188 pl = 4.18 nl
225 $\mu$	5,900,000 $\mu^3$	=5900 pl = 5.90 nl
250 $\mu$	8,203,000 $\mu^3$	=8203 pl = 8.20 nl
275 $\mu$	11,037,000 $\mu^3$	=11037 pl = 11.04 nl
500 $\mu$	65,450,000 $\mu^3$	= 65 nl
1000 $\mu$ = 1mm	523,600,000 $\mu^3$	= 0.52 $\mu\text{l}$
2000 $\mu$ = 2mm	4,200,000,000 $\mu^3$	= 4.2 $\mu\text{l}$