Parental RNAi

(Gregor Bucher & Martin Klingler 8/02, with input from Sue Brown and Andrew Fire lab;
please cite: Bucher et al. 2001, Current Biol. R85-R86

Prepare injection apparatus (simple XYZ-micromanipulater) and a binocular; the capillary holder should be oriented at an angle of approximately 60 degrees

Select 20-30 female pupae; use older puae (eyes pimented, wings greyish) because they survive treatment better (if you plan to fix the eggs for stainings, you may want to inject more pupae - 50 or so).

Bring rubber cement (Fixogum) onto a microscope slide.
Glue pupae with their posteriormost(!) part of the abdomen to the glue (gluing more anteriorly will prevent them from hatching!), such that the ventral side is up.

Break glass capillary such that the tip is about as thick as the sclerotized part of the urogomphi.

Take up 2-4 ul of dsRNA solution (RNA in Anrew Fire's injection buffer, coloured by adding 1/10 volume of PhenoleRed solution, Sigma P-0290) into a yellow Eppendorf tip. Fix yellow tip to microscope slide with paraplast, fill injection capillary by immersing the tip into the opening of the yellow tip and applying vacuum (syringe). Be carefull not to aspirate air.

Inject pupae ventro-laterally between abdominal segments 3 and 4 (just posterior of wing tips; injection into the ventral midline results in higher mortality). Stop injecting when pupae stretch due to increased haemolymph pressure. The solution can be seen pinkish through the body wall - it will extend anteriorly into the abdominal cavity and may initially be restricted to one side of the pupa but will subsequently distribute evenly.

Place the injected pupae (still attached to the slide) upside down on full grain flour such that pupae inmerse in flour and the slide "covers" them. Add males or male pupae. After several days at 33°Celsius, remove the slide (you may have to free some beetles using a brush). Pupae have to eat full grain flour for some days in order to lay eggs!

The first egglay can be taken 7 days post-injection, althoug maximal egg production is reached a bit later. The portion of phenocopies initially is high (up to 100%) but drops within 4-5 weeks to zero. It is very important, therefore, to collect during the first two weeks (with strongest phenotypes and highest penetrance in the first week).

dsRNA concentration: use at least 2ug/ul - the more the better (up to 6ug/ul; high concentrations can result in sterility, though)

Generation of dsRNA

For generation of Template use the following PCR primers (primer combination according to template plasmid used, i.e. bluescript: T7 & T7-T3, pZero: T7 & T7-SP6):
T7: 5' gaa ttg taa tac gac tca cta tag g 3'
T7-T3:5' taa tac gac tca cta tag gaa tta acc ctc act aaa ggg 3' (anneals to T3 promotor and has a T7 promotor sequence attached)
T7-SP6: 5' taa tac gac tca cta tag gat tta ggt gac act ata ga 3' (anneals to SP6 promotor and has a T7 promotor sequence attached)
SP6 (for in situ ssRNA templates): 5' gat tta ggt gac act ata ga 3'
T3 (for in situ ssRNA templates): 5' aa tta acc ctc act aaa ggg 3'

1. PCR:

H2O + DNA	13,8ul (5 to 50ng DNA - too much can inhibit PCR!)	
10Xpuffer	2	
dNTP	2	
Primer T7	1	
Primer T7-Sp6/T3	1	mix reaction
Taq Polymerase	0,2	mix again
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	20 ul	

PCR program:

1'	94°		
30''	94°	*	30X
1'	40° für dsRNA, 50° für Sonden	*	30X
1'	72° (one minute per kb template length)	*	30X
2'	72°		
constant 4°			

2. precipitation:

add to reaction:

3M NaAc pH 5,2	2ul	
Glycogen (carrier, Roche)	1ul	mix
EtOH 100%	50ul	mix

-20° for 1h; centrifuge 1h (15.000);
wash pellet with 500ul EtOH 70%, centrifuge 30', discard supernatant, air dry pellet
dissolve in 10ul H2O (pipetting up and down, the shake 15' on eppendorf mixer)
determine concentration: dilute 1ul 1:10 and bring 1 and 5 ul on a gel. (5ul band should have 25ng or more, such that 500 ng or more template can be used for in vitro transcription)

3. In vitro transcription for dsRNA (RNAi):

we use the T7 Ambion Megascript Kit (since the template has T7 promotors at both ends, only one enzyme, i.e. T7, is required; annealing of sense and antisense RNAs occurs during the in vitro transcription reaction, no additional annealing procedure required)

be careful to fully resuspend reaction buffer and rNTP solutions; assemble the reaction at room temperature to avoid precipitation of certain components at 4°C

template (PCR product):	8ul	
10X buffer	2ul	
nucleotides each	2ul	
	2ul	
	2ul	
	2ul	mix
enzyme-Mix	2ul	mix

	20ul	keep at 37° for 3 hours (not over night)

(In vitro transcription for in situ probes :

for antisense probe use template where only the 3' primer includes the T7 sequence - the other primer should be T3, SP6 or M13/M13rev ...)

4. LiCl-Fällung:
add to reaction (which may look a little milky):

H2O	30ul
LiCl-Lsg	25ul (mix)

precipitate at -20° for 1h (freezes)

thaw, centrifuge for 30' at 15.000 rpm
remove supernatant (a milky pellet should be visible)
add 500ul 70% EtOH
centrifuge again for 30' at 15.000 rpm,
remove supernatant, let dry at air
resuspend in 50ul annealing puffer (potassium phosphate 20mM, sodium citrate 3mM, pH7.5; Fire, Xu et al. 1998).

5. determine conzentration (for RNAi):
dilute 1ul in 100 ul dH2O
measure OD (RNA-settings!!)
if OD is 10-20 ug/ml, concentrated stock is 1-2 ug/ul, i.e. total yield 50-100 ug RNA per reaction