DsRNA from a PCR-template

(Gregor Bucher, Martin Klingler, 4/05)

For dsRNA use the following primers:
1) T7
2) T7-T3 (anneals to T3 promotor and has a T7 promotor sequence attached)
3) T7-SP6 (anneals to SP6 promotor and has a T7 promotor sequence attached)
4) Use primer combination according to template plasmid used (bluescript: T7 & T7-T3, pZero: T7 & T7-SP6) or

1. PCR:

H_2O + DNA	24,6 (Plasmids: just dip in 5mm with a tip, stir twice and inoculate reaction or: 0,2 ul of a 1:100 dilution or 10ng - too much inhibits PCR!)	
$MgCl_2$ (25mM)	2,4	(1,5 mM endconcentration)
10Xpuffer	4	
dNTP (2mM)	4	
Primer 1	2	
Primer 2	2	mix reaction
Taq Poly (Fermentas)	1	mix again
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	40 ul	

PCR program:

3'	94°		
30''	94°	*	
30''	60°	*	30X
2'	72° (one minute per kb template length)	*	
3'	72°		
constant 8°			

2a with precipitation:

3M NaAc pH 5,2	4ul	
EtOH 100%	80ul	mix

-20° for 1h; centrifuge 1h at 4°C (max);
wash pellet with 500ul EtOH 70%, centrifuge 30' at 4°, discard supernatant, air dry pellet
dissolve in 20ul H_2O (pipetting up and down, wash also sides of eppi!), let evaporate residual EtOH by placing open eppi in 50°C heating block for 3-5 min.
determine concentration (usually 300-400 ng/ul).

3. In vitro transcription for dsRNA (RNAi):

use the T7 Ambion Megascript Kit (since the template has T7 promotors at both ends, only one enzyme, i.e. T7, is required)

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

template + water	8ul	(PCR product: 300-500ng)
10X buffer	2ul	
nucleotides each	2ul	
	2ul	
	2ul	
	2ul	mix
enzyme-Mix	2ul	mix

	20ul	keep at 37° for 4-5 hours (not over night)

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

H_2O	30ul	
LiCl-Lsg	25ul	(mix)

precipitate at -20° for 1h (freezes)

- thaw, centrifuge for 30' at 15.000 rpm at 4°C
- remove supernatant (a milky pellet should be visible)
- add 1ml 70% EtOH
- centrifuge again for 30' at 15.000 rpm at 4°C,
- remove supernatant, let dry at air for 20-30 min → It's important to dry the pellet very carefully because residual LiCl will lead to a very low survival rate!!!

resuspend in 20-40 ul injection puffer (H2o works also, but solution is less viscous in buffer – especially useful for eRNAi; repeated freezing and thawing may help bring the RNA into solution)

[5. annealing
(this procedure changes the product as observed on the gel: the smear becomes less pronounced and the intensity focusses in a smaller region. This might indicate higher portion of dsRNA. We have not checked for changes in interference effect. Most preparations also work without annealing – we have not compared efficiency)

(prepare 100 ml boiling water, place Eppi for 2 minutes on 95° heating block, remove water from stove, place Eppi in the water bath until temperature is about 70° (15 minutes).)

place 5´in 94° heating block, take out block with eppi and let cool at room temperature for 40´.]

6. determine conzentration (for RNAi):

nano-drop: 1ul
measure OD (dsRNA-settings are 45 (info by nano-drop))
concentration should be 1-5 ug/ul – at higher concentrations viscosity is too high for injection)

7. injections:
remove contaminating particles (which might clogg injection capillary) by centrifugation 1´ maximum (alternatively: use 45um Ultrafree-MC (Millipore) – but you will loose volume). For injection of pupae/adults you may omit this step – because the capillary is thicker

Inject as much as possible (2 - 5 ug/ul). Also lower concentrations (100-500 ng/ul) can result in phenotypes, but usually weaker.

Primer sequences:

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'
T7-SP6: 5' taa tac gac tca cta tag gat tta ggt gac act ata ga 3'
T7-M13R: 5' taa tac gac tca cta tag gca gga aac agc tat gac 3'

for in situ ssRNA templates:

SP6: 5' gat tta ggt gac act ata ga 3'
T3: 5' aa tta acc ctc act aaa ggg 3'
M13R: 5' cag gaa aca gct atg ac 3'

injection buffer:

1,4 mM NaCl
0,07 mM Na2H PO4
0,03 mM KH2 PO4
4 mM KCL

Parental RNAi

(Gregor, 9/06)

Prepare injection aparatus and a binocular; the needle should be oriented in angle of approximately 60 degree.

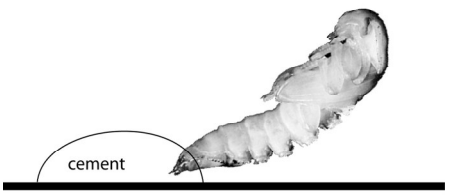
The angle of the needle is approximately 60 degree, pressure is controlled by a 12 or 50 ml syringe.

Select 10-20 female pupae; use old pupae (dark wings, eyes developed) because they survive treatment better (if you plan to fix the eggs, you may want to inject 50-200 pupae).

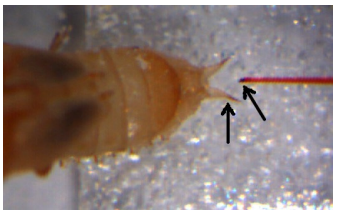
Fix double sided sticky tape along one rim of a microscope slide. (Alternative: Bring rubber based cement (Fixogum) onto a microscope slide)

Glue pupae with their posteriormost dorsal part of the abdomen to the glue (ventral side with wings is up). The region should not exceed the size of the last sternite. This is a critical step: gluing more anterior body parts will prevent them from hatching!

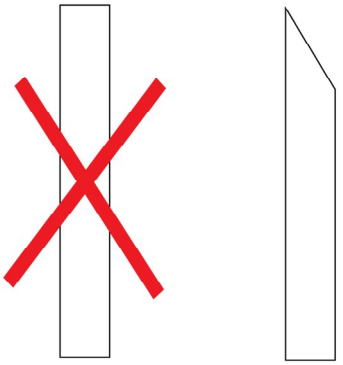
This is a pupae shortly before hatching (dark wings, legs sclerotized, eyes fully developed)



Side view of the slide and the rubber based cement. If you glue more of the abdomen to the cement (or sticky tape) the survival rate goes down.



Break capillary such that the tip is approximately as thick as the sclerotized part of the urogomphi (see arrow). This is not very critical for survival but influences the handling. (Too much of the abdomen has been glued to the sticky tape – this pupa will not hatch)



It's important that the tip of the capillary is sharp to simplify the injection.

Pipet 5 ul of dsRNA solution onto an upside down Eppi-cap. Fill capillary through the tip by immersing it into the drop and applying slight underpressure. Be careful not to aspirate air – the dsRNA may suddenly be sucked into the capillary holder.

Inject laterally between abdominal segments 3 and 4 (just where the wing tips point to) (central injection leads to higher mortality). Go in by one fast movement (reaching appr. the middle of the pupa) then go back a bit. If fluid doesn't flow, move needle up and down a bit. Stop injecting when pupae are stretched and cannot move anymore because of raised turgor. When leaving the pupa you have to reduce the pressure at the same time (if not, the solution will leak out – if you reduce too much, solution will be sucked into the capillary holder…). Solution will initially extend anteriorly into the abdominal cavity and may be restricted to one half, but will distribute subsequently. The rule is: the more you inject the better.

The left pupa is not injected, the right one is stretched because of the filling (PhenolRed).

Place the injected pupae together with the slide upside down on full grain flour such that pupae inmerse in flour and the slide "covers" them. Add males or male pupae (5 males for 20 females). After 4-5 days at 32° Celsius remove the slide (you may have to free some beetles from the glue).

Females need to feed on full grain flour before they can lay eggs and need appr. two days after hatching before they start to lay eggs.

Depending on the age of pupae, the first egglay can be taken 5-7 days post injection although maximal egg production is reached later. The portion and strength of phenotypes is high initially (up to 100%) but drops within days or weeks to zero (depending on gene and amount of injected dsRNA). Also the strength usually drops strongly with time. It is thus important to collect the first days.

DsRNA solution: use up to 1-5ug/ul (with higher concentrations, the solution gets too viscous for injection). The strongest effects are usually created by embryonic injections (up to 2ug/ul)!