Preparation of dsRNA from PCR products (single tube synthesis)

Adapted from Kafatos lab using MEGAscript T7 Kit (Ambion)

1. PCR

• Design PCR primers for a 200 – 600 bp section of the gene of interest. The targeted region should not be located in high homology regions, and preferably located as close to the 3' prime end of the gene as possible. Tail both your PCR primers with the T7 promoter sequence GAATTAATACGACTCACTATAGGGAGA at their 5' end. Use this website to assist you (http://www.dkfz.de/signaling2/rnai/).
• Standard purification (desalt) of the primers is acceptable.
• Amplify the fragment by PCR using the following concentrations:

10x PCR Buffer	5	µl
$MgCl_2$	2	µl
Platinum Taq Polymerase	0.2	µl
dNTP mix (2.5 mM)	4	µl
Genomic DNA template (250ng/µl)	1	µl
Primer Mix (10 pmol/µl)	2	µl
ddH2O	36	µl
Total:	**50**	**µl**

Note: cDNA should be used as PCR template, but genomic DNA (at 1 µg/µl) is acceptable if the targeted region does not contain an intron and you are confident of the gene architecture.

2. Purification of PCR product (If there are primer dimers and non-specific products). The purification of the PCR product is achieved with the QIAquick PCR Purification kit (QIAGEN) or Gel extraction Kit.

3. Production of dsRNA

The purified PCR amplicons are now used to synthesize dsRNA with the **MEGAscript T7 Kit or MEGAscript RNAi Kit(Ambion)**.
• Add to an RNase free tube in this order:

ATP	2	µl
CTP	2	µl
GTP	2	µl
UTP	2	µl
Buffer (warm to 37˚C)	2	µl
cleaned PCR product	8	µl
Enzyme mix	2	µl
Total	**20**	**µl**

• Mix gently and spin down.
• Incubate at 37˚C for 6-12 hours (preferably with constant shaking). Incubation times may vary – check viscosity of the sample by tapping on the tube with your finger – its viscosity will increase with time.
• Incubate at 75˚C for 5 min (Anneal), then cool down to room temparture (~25˚C) for about 3~4 hr or longer. Freeze dsRNA at -20˚C or -80˚C.

4. Digestion of dsDNA and ssRNA using MEGAscript RNAi Kit(Ambion)

Nuclease-free water	21	µl
Digestion buffer	5	µl
dsRNA	20	µl
DNase I	2	µl
RNase A	2	µl
Total	**50**	**µl**

5. Purification of dsRNA

Nuclease-free water	150	µl
dsRNA	50	µl
Phenol	100	µl
Chloroform	100	µl
Total	**400**	**µl**

• Vortex, full spin at 4 ˚C for 10 min.
• Transfer 200 µl supernatant to a new tube. Add 20 µl 3M sodium acetate (p.h.=5.2) and 600 µl 100% ice-cold ETOH. Mix well and store at -80 ˚C for about 20 min.
• Full spin at 4 ˚C for 15 min. Wash the pellet with 500 µl of 70% ETOH and full spin at 4˚C for 5 min.
• Air dry and add 15 µl nuclease-free water. Then dilute 1 µl of dsRNA ten times. Measure dsRNA concentration with Nanodrop spectrophotometer and check it on an 1% agarose gel.

note: The dsRNA is not denatured and annealed by a boiling and cooling cycle.