RNA preparation and annealing for dsRNA injection
adapted by Erik Andersen (January 16, 2006)

I. PCR amplify gene of interest:

Using primers that flank the polylinker region, setup a standard 100 μL PCR.
For example, to amplify a cDNA in a BlueScript SK+/- vector, use the following primers:

oBSF 5' ttg taa aac gac ggc cag 3'
oBSR 5' cat gat tac gcc aag ctc 3'

oBSF and oBSR flank the T7 and T3 promoter sequences. The amplified PCR product has T3 and T7 promoter regions flanking the gene of interest. This amplification protocol enables synthesis of a + or - RNA strand by using T3 or T7 polymerase in the *in vitro* transcription reactions (IVTs). The amplified PCR product can be used directly (without phenol/chloroform extraction) for *in vitro* RNA transcription. The quality of template should be checked on a 1% agarose gel. If the PCR product is a bright single band, use ~20% of PCR mix for the IVT.

NOTE: These primers work for all yk less that yk700 and cm clones from the database project. For yk clones greater than yk700 you must use primers containing T3 and T7 sequences to amplify the clone. I use:

oMEF_T3 5' aattaaccctcactaaagggcttctgctctaaaagctgcg 3'
oMER_T7 5' taatacgactcactatagggtgtgggaggttttttctcta 3'

Both T3 and T7 must be used to synthesize ssRNA, because later you want to anneal roughly equimolar ratios of T3 and T7 transcribed products. This way you can control for the production of both transcripts. Using only T7 to synthesize both strands cannot control for how much of each strand you have produced and then added to the annealing reaction.

II. *in vitro* transcription reaction:

5-10 μg of DNA (20 μL of total PCR template)
20 μL 5X transcription buffer
20 μL 10mM ATP, CTP, GTP, UTP NTP mix from Invitrogen
10 μL 0.1M DTT
1 μL RNAsin (RNAse inhibitor from Promega)
27.75 μL RNAse-free water (MilliQ water is fine)
1.25 μL T3 or T7 polymerase (high-concentration enzyme from Invitrogen)

1. Mix by pipeting gently and incubate at 37ºC for two to two and a half hours.
2. Add 5U of DNAse. Mix gently by pipeting and incubate at 37ºC for 15 minutes.
3. Add 1X volume of phenol:chloroform:isoamyl alcohol 25:24:1 and vortex vigorously for 30 seconds.
   ****Be careful to not pickup any of the Tris overlay****
4. Spin at maximum speed for two minutes (room temp.)
5. Pipet out the bottom layer, and put it into the phenol:chloroform liquid waste.

****Be careful not to remove top and interface layers.****

6. Optional: Add 1X volume of phenol: chloroform: isoamyl alcohol 25:24:1
7. Optional: Vortex for 30 seconds and spin at maximum speed for three minutes (room temp). Remove bottom layer.
8. Add 1X volume of chloroform.
9. Vortex for 30sec and spin at max speed for four minutes (room temp).
10. Transfer the top layer to 1.5 mL microfuge tube. Be careful to transfer only the top layer.
11. Add 10% volume of 3M sodium acetate pH 5.2 (usually 10 μL) and 0.6 volume of isopropanol (usually 60 μL).
12. Mix well by vortexing and spin at max speed for seven minutes (room temp) and resuspend pellet in 20 μL RNAse-free 1X injection buffer.
13. Check the quality of synthesized RNA by running ~4 uL of IVT mix next to PCR template. *This step serves two purposes: 1) enables one to check how good is the RNA, and 2) By running it next to its DNA template allows one to roughly double check the relative size of the ssRNA. ssRNA often runs as a smear on a standard agarose gel. Do not be alarmed.

III. Annealing the ssRNAs to make a dsRNA for injection:

1. Mix equimolar ratios of the T3 and T7 synthesized ssRNAs. I use the remaining synthesis reaction left after running out to check the single-stranded products.
2. Heat to 85ºC for 30 minutes.
3. Heat to 37ºC for 30 minutes.
4. Cool on benchtop for 30 minutes. Spin down at max speed.
5. Run out 4 μL to check the dsRNA. It should run like “smeary” PCR product at roughly the same size. It should look less “smeary” than the ssRNA products.
6. Store at -20ºC until use and keep on ice while loading needles.
7. I mouth-pipette the dsRNA solution into the pulled needles because the solution is quite viscous. Draw out a 20 μL pipette under a flame to get a fine tip and insert the tip into the pulled needle until it is next to the taper of the needle head. Dispense the dsRNA here and carefully remove the pipette from the needle.