Protocol for preparation of oocyte cRNA

1) Linearize the oocyte vector containing your gene of interest. Use a restriction enzyme site that is located 3' of your gene and after the termination site. Make sure the restriction enzyme site does not cut within your gene sequence. Linearize 10-20 μg of DNA.
2) Run 5 μl of the reaction on a DNA agarose gel to confirm that the vector is fully linearized. The presence of even a small amount of circular vector will greatly decrease cRNA yield.
3) Treat the linearized DNA with proteinase K to remove any RNases that might be present. For a 50 μl reaction:
    a. Add 5 μl of 10% SDS to each reaction.
    b. Add 2 μl of proteinase K (Roche)
    c. Incubate at 50°C for 30 min.
4) FROM THIS POINT ON EVERYTHING MUST BE RNASE FREE.
5) Purify the DNA by phenol extraction.
    a. Combine your restriction digest reactions if you have more than one of the same vector
    b. Add DEPC-H_2O to make the final volume 500 μl.
    c. Add 500 μl of phenol and vortex thoroughly.
    d. Centrifuge at 13,000 rpm for 2 min.
    e. Remove the top layer and place into a new tube. Do not disturb the intermediate protein band or the lower layer. Discard these layers.
    f. Add 250 μl phenol and 250 μl chloroform and vortex.
    g. Centrifuge at 13,000 rpm for 2 min.
    h. Remove the top layer and place into a new tube. Discard the bottom layer. If the protein band is present at this stage, be careful not to disturb it, and also repeat steps f-h again. If the protein band is not present, proceed to step i.
    i. Add 500 μl of chloroform and vortex.
    j. Centrifuge at 13,000 rpm for 2 min.
    k. Remove the top layer and place into a new tube.
    l. Add 1/10 the total volume of 3M NaAcetate and mix.
    m. Add 2 volumes of 100% ethanol and mix.
    n. Precipitate at -20°C overnight (preferred), or at least 1 hour.
    o. Spin at 13,000 rpm at 4°C for 30 min.
    p. Remove ethanol without disturbing the pellet.
    q. Add 500 μl of chilled, 70% ethanol.
    r. Spin at 13,000 rpm at 4°C for 5 min.
    s. Remove ethanol without disturbing the pellet and let dry for ~5 min.
    t. Resuspend the DNA in 20 μl of DEPC-H_2O.
6) Determine the concentration and purity of the DNA using the nanodrop.
7) Using the mmachine transcription kit (Ambion) set up the RNA transcription reaction. Use the kit containing the RNA polymerase for your specific vector (either T7, T3, or SP6). Use non-stick tubes. Add in the following order:

a.	DEPC-H_2O	to 20 μl
b.	2X NTP/CAP Buffer	10 μl
c.	10X Buffer	2 μl
d.	Purified, linear DNA	1-2 μg
e.	GTP (if construct is >7.5kb)	1 μl
f.	Enzyme mix	2 μl

8) Incubate the reaction at 37°C for 2 hours.
9) Add 1 μl turbo DNase mix and incubate at 37°C for 15 min.
10) Add 115 μl DEPC-H_2O and 15 μl Ammonium Acetate stop solution
11) Do a phenol extraction
    a. Add 150 μl of phenol and vortex.
    b. Spin at 13,000 rpm for 2 min.
    c. Remove the top layer and place in a new tube.
    d. Add 75 μl of phenol and 75 μl of chloroform and vortex.
    e. Spin at 13,000 rpm for 2 min.
    f. Remove the top layer and place into a new tube.
    g. Add 150 μl of chloroform and vortex.
    h. Spin at 13,000 rpm for 2 min.
    i. Remove the top layer and place into a new tube.
    j. Add an equal volume of isopropanol to precipitate the cRNA.
    k. Precipitate at -20°C overnight (preferred), or at least 2 hours.
    l. Spin at 13,000 rpm at 4°C for 20 minutes.
    m. Remove the isopropanol without disturbing the pellet.
    n. Resuspend in chilled, 70% ethanol.
    o. Spin at 13,000 rpm at 4°C for 5 min.
    p. Remove ethanol and let dry 3-5 min. The pellet should look glassy. Take care not to let the RNA pellet dry completely! If the pellet dries completely, it will be extremely difficult to resuspend the RNA and the yield will be decreased considerably.
    q. Resuspend in DEPC-H_2O. The RNA can be incubated for about 5 minutes at 50°C to facilitate resuspension if necessary.
12) Measure the concentration and purity (260/280) ratio. The ratio should be > 1.8.
13) Run the cRNA on a gel to ensure the presence of a sharp band. Often, the cRNA will run as a double band due to the formation of secondary structures. A smear indicates degradation. The size of the cRNA cannot be determined on the gel unless a denaturing gel is used.
14) Aliquot the RNA into 3 μl aliquots.
15) Freeze and store at -80°C.