In vitro transcription protocol

1. **PCR with appropriate primer set to obtain cDNA fragment flanked by RNA polymerase binding sites**.
We generally run 25 ul PCR reactions.

2. **Check PCR product size and quantity on agarose gel**.
Run 2.5 ul on a normal agarose gel

3. Precipitate PCR product.

20 ul of PCR product (see note)
10 ul 3M Sodium Acetate (pH5.5)
70 ul water
900 ul cold 100% ethanol

-incubate at -20C for 30 minutes and spin at max speed for 30 minutes
-remove ethanol from RNA pellet and wash pellet with 70% ethanol (500ul).
-resuspend in 10 ul of DEPC water

Note: the goal is to have 1ug of DNA template, however in practice this much is rarely needed to make a good probe. 20 ul of a DNA sample that shows a decent intensity band on a gel will be fine. You may have to use your own judgment.

4a. **In vitro transcription reaction T7 or SP6 (we use NEB enzymes)**:

PCR product	10 ul	(use 1 ug of DNA in DEPC dH20)
10X transcription buffer	2 ul	
10X DIG labeling mix	2 ul	(Roche, contains DIG-UTP)
RNasin (40U/ul)	0.5 ul	(Promega, prevents RNase activity)
RNA polymerase (15-17U/ul)	1 ul	(T7 or SP6)
DEPC dH_2O	4.5 ul	
	20 ul	

-incubate 2-3 hours at 37ºC.
-add 1 ul of RNasin (40U/ul) and 1ul of RQ1 DNase.
-incubate 30 minutes at 37C. can store at –80ºC.

4b. **In vitro transcription reaction T3 (we use Promega enzyme)**:

PCR product	10 ul	(use 1 ug of DNA in DEPC dH20)
5X transcription buffer	4 ul	(Promega)
0.1M DTT	2 ul	
10X DIG labeling mix	2 ul	(Roche, contains DIG-UTP)
RNasin (40U/ul)	0.5 ul	(prevents RNase activity)
RNA polymerase (15-17U/ul)	1 ul	(T3)
DEPC dH_2O	0.5 ul	
	20 ul	

-incubate 2-3 hours at 37ºC.
-add 1 ul of RNasin (40U/ul) and 1ul of RQ1 DNase.
-incubate 30 minutes at 37C. can store at –80ºC.

Note, the protocol is the same for biotinylated probes. Just substitute Roche biotin RNA labeling mix in the above protocol.

5. **Check RNA agarose gel (1%)**

We normally run 1-2 ul of the RNA reaction on a gel to examine 1) if RNA was made and 2) to get a rough idea of the concentration. Running a formaldehyde gel is optional, regular gels work just fine for this purpose. Normally, we run the gel while performing step 6. However, the RNA reaction can be stored at -80C while you run the gel and then you can proceed to step 6 once you are confident about the probe reaction working.

6. **Probe fragmentation and precipitation**.

Some protocols suggest that the optimal fragment size for RNA hybridization is 500bp. We have found that hydrolyzed probes work slightly better than non-hydrolyzed probes. If your probe is <1kb bp is not a good idea to hydrolyze as your fragments will be very small. Hydrolysis of larger probes (>1kb) can be achieved by using a mild alkaline solution as follows:

-add an equal volume (20 ul) of 0.2M Na_2C0_3 pH 10.2 to each probe.
-incubate at 60ºC for 15 minutes
-Place on ice, add 20ul of 7.5M NH_4OAc and 160 ul of 100% ethanol.
-incubate at RT for 10 minutes and spin at max speed for 30 minutes
-remove ethanol from RNA pellet and wash pellet with 70% ethanol (500ul).
-resuspend in 150 ul of resuspension buffer and store at –80ºC.

Solutions:
3M Sodium Acetate (pH 5.5)
100% and 70% Ethanol
0.2M Na_2C0_3 (pH 10.2)
7.5M Ammonium Acetate
Resuspension buffer: 50% Formamide, 5mM Tris (pH7.5), 0.5mM EDTA, 0.01% Tween

Roche Dig RNA labeling Mix
T7, T3, and SP6 enzymes and accompanying buffers