Probe Preparation

Cut 20-40 µg of plasmid DNA

I. Digest Rxns:

DNA	10-16µl
RE (20U/µl)	1µl
10X Buffer	2µl
20X BSA	1µl
dH2O	To volume
Total volume	**20µl**

-Incubate O/N at 37˚C

Phenol/Chloroform Extraction

1. Extract DNA from samples by adding an equal volume of phenol/chloroform at 25:24 (bring samples to 450µl first to minimize DNA loss)
2. Vortex for 10 sec and spin for 5' at 12400xg (4C)
3. Transfer aqueous phase to a clean tube, add 450µl of chloroform, vortex for 10 seconds, and spin for 5' at 12400xg (4C).
4. Transfer the aqueous phase to a clean tube, add 1/10 volume NaOAC pH6.0 and 2.5x volume 100% EtOH
5. Invert several times and put in -80˚C for 30'
6. Spin at 4˚C at 12,400xg for 15'
7. Remove 100% EtOH and add 500µl of 70% EtOH
8. Spin for 10' at 15,000xg at 4˚C
9. Remove EtOH, dry the pellet, and resuspend in 12µl DEPC dH_2O

OR

I. PCR:

Sigma H2O	38.25µl
10X Titanium buffer	5µl
dNTP(10mM)	1µl
Plasmid DNA (1-15ng)	0.5µl
T7(10µM)	2.5µl
T3 or Sp6(10µM)	2.5µl
50X Titanium Taq	0.25µl

cycling parameters: 95˚C for 1min, 35 cycles: 95˚C for 30sec, 55˚C for 30sec , 68˚C for 1min/Kb

II. Transcription:

10X buffer	5µl
T7,T3, or Sp6(20U/µl)	4.5µl
Rnasin (40U/µl)	1µl
Dig mix	5µl
1-2.5 µg linearized DNA	2-10µl
DEPC dH_2O	volume
Total volume	**50µl**

OR

10X buffer	5µl
T7,T3, or Sp6(20U/µl)	4.5µl
Rnasin (40U/µl)	1µl
Dig mix	5µl
PCR DNA	10µl
DEPC dH_2O	24.5µl
Total volume	**50µl**

1. Incubate samples at 37˚C for 2 or more hours
2. Add 2µl (20U) DNase1 to each reaction and incubate at 37˚C for an additional 10min
3. Add 52µl of stop buffer to each reaction
4. To remove access Dig-labeled nucleotides transfer each sample to a Sephadex G50 column:
    a. Add 800µl of DEPC dH2O to the column after tapping it to ensure that the gel powder is on the bottom (also make sure to leave the end stopper on)
    b. Replace the cap, vortex briefly, and incubate at RT for 30min
    c. After incubating invert the column, tap sharply, and return to an upright position. This is to remove air bubbles
    d. Remove the stopper and allow column fluid to flow into the catch container (~200-250µl). It may be necessary to spin at 750xg for a few seconds
    e. Discard the flow-through and spin the columns at 750xg for 2min at 4˚C.
    f. Remove the catch tube and put the column into a clean tube, add the entire transcription reaction directly onto the top of the gel in a drop-wise fashion. Do this quickly so that the gels do not dry out
    g. Spin the tubes at 750xg for 2min at 4˚C being sure that the tubes are oriented in the same way as the first spin

5. After spinning EtOH precipitate the probes:
    a. add 1/10 volume NaOAC pH6.0 and 2.5x volume 100% EtOH
    b. Invert several times and put in -80˚C for 15min
    c. Spin at 4˚C at 12,400xg for 15min
    d. Remove 100% EtOH and add 500µl of 70% EtOH
    e. Spin for 5min at 20,000xg at 4˚C
    f. Remove EtOH, dry the pellet, re-suspend in 50µl DEPC dH_2O
6. OD the probes and then dilute them to 1µg/ml in hybridization buffer
7. Remove 1µl of each sample to run on a 1% gel to check for correct probe MW.