Synthesis of high specific activity cDNA probe using ^{32}P

Perform the first stage of the reaction using RNAse-free reagents and plasticware. Plasticware is generally RNAse free if it has not been touched and then autoclaved (or irradiated). Keep RNA thawed for the minimum time possible; i.e. don't thaw tube from freezer (-80°C) until last minute, and keep tubes on ice until you start the reaction.

1. Annealling of primers

Either oligo-dT or random hexamers can be used to make cDNA. For probes, maximum activity results from using as primers random hexamers, or hexamers + polyT, compared to polyT alone.

total volume = 5 µl
0.5 to 2 µg poly A^+ RNA
500 ng oligo$(dT)_{18}$ *(or equivalent)*
150 ng random hexamers
RNAse-free water (i.e. DEPC treated and autoclaved) to 5 µl *total* volume.

Heat 70°C for 2 min.
Place on ice for 2 min.

2. Labelling

Add to the annealed primer-RNA mixture:
10 µl of 10 mC/ml ^{32}P-dCTP (100 µCi total; stock is >3000 Ci/mM)
0. 8 µl of 1 mM unlabelled dCTP (final is 20 µM)
0.8 µl of 10 mM unlabelled dATP, dGTP, dTTP (mixture of the 3, 30mM dNTP total); final is 200 µM final
8 µl 5X labelling buffer (5X is 50 mM Tris-Cl pH 7.5, 75 mM KCl, 3 mM $MgCl_2$; this is the standard buffer from Invitrogen/Life Technologies)
4 µl Reverse transcriptase (Superscriptase from Life Technologies)
5 µl water (to 40 µl final volume)

Incubate 42°C, 1 hour

3. Hydrolysis of RNA/Stopping the reaction

Add 40 µl of 0.8 M NaOH, 20 mM EDTA
Incubate 65°C, 30 min.

4. Checking incorporation.

Making cDNA probes is not a foolproof as making a standard probe, so it always helps to perform this step to ensure that you have a "good" probe.

Take 1 µl cDNA reaction product.
Add 22 µl of 20 mM EDTA and 2.5 µl of tRNA stock.
Spot 10 µl on glass fiber filters.
Air dry
Wash the filters, along with a blank filter as a control, as follows:
Wash 3 times, 10 min each, with 50-100 ml cold 10% trichloroacetic acid (TCA).
Carefully decant each of the wash solutions into the waste container.
Wash 1X with 95% ethanol.
Dry.
Place filters in scintillation tube.
Add 3-5 µl scintillation fluid.
Count. A good probe should be about 1-2 x 10^8 cpm.

5. Cleaning up the probe for blot (removing unincorporated label, which helps reduce background)

The method is the same as for a DNA probe, and involves running the sample over a P-60 gel filtration column:

a. Make a minicolumn in a narrow-tipped plastic transfer pipette.
Cut off the bulb (top)
Plug the bottom using a small chunk of sterile, siliconized glass wool.
Support the column in a 2 ml screw-cap tube.
Add gel slurry (50-66% Biogel P-60, 100-200 mesh, in TE plus 0.2 % SDS).
Add about 2 inch of 66% slurry for a typical column (1-1.2 inch final height, once the resin is settled)
Wait for the resin to settle and for the liquid to drain out; discard the liquid.

b. To the DNA labelling reaction (50 µl) add 20 µl of column loading buffer:
50% glycerol
0.1% bromophenol blue (dark blue)
1% blue dextran (light blue-aquamarine)
(store a stock of this at 4C; aliquots can be kept at room temp for 2+ months)

c. Load the reaction on the column. For the greatest efficiency, try to squirt it down to the top of the resin, against the side of the column, in one squirt.

d.Once the reaction has entered the resin, fill the column with TE-0.2% SDS.

e. The labelled DNA will elute before the bromophenol blue elutes, with and just behind the blue dextran. Save all of the liquid that comes out before the bromophenol blue. Discard the column. Cap the tube.
The column flow-through (ssDNA) can be added to the blot directly, without boiling like a normal DNA probe (which is dsDNA).