with RNase-free DNase or use more mRNA sample.

2. Some populations of mRNA molecules may have tight secondary structures. Methylmercuric hydroxide treatment of the RNA sample may be necessary. Perform the following protocol under a fume hood. Resuspend the mRNA in 20 µL of DEPC-treated water and incubate at 65°C for 5 min. Cool to room temperature and add 2 µL of 100 m*M* methyl-mercuric hydroxide. Incubate at room temperature for 1 min, add 4 µL of 700 m*M* β-mercaptoethanol (dilute stock in DEPC-treated water), and incubate at room temperature for 5 min. The final volume is 26 µL. This denatured mRNA is ready for first strand synthesis.
3. Do not use [α-^{32}P]dCTP. The 5-methyl dCTP present in the nucleotide mixture will be diluted and the synthesized cDNA will not be protected from the subsequent restriction digest. Gel analysis may show a false negative result if the [α-^{32}P]dNTP is degraded, because it may not incorporate into the cDNA even though synthesis is occurring.
4. Gel analysis may show hairpinning of the cDNA, which is caused by a number of factors: an insufficient amount of mRNA was used in the first strand reaction (*see* **Note 1**); the mRNA population had tight secondary structure (*see* **Note 2**); the second-strand incubation temperature was higher than 16°C (cool the first strand reaction by placing it on ice before adding the second strand synthesis reaction components); or an excessive amount of DNA polymerase was used in the second strand reaction.
5. Phenol:chloroform (1:1 [v/v], pH 7.0–8.0) is recommended. Do not use low-pH phenol routinely used for RNA isolation ***(1,2)***. To extract the cDNA sample, add an equal volume of phenol:chloroform (1:1 [v/v], pH 7.0–8.0) and vortex. Microcentrifuge at maximum speed for 2 min. Transfer the upper aqueous layer, which contains the cDNA, to a new sterile tube. Avoid removing any interface. Add an equal volume of chloroform, and vortex. Microcentrifuge for 2 min at maximum speed. Save the upper aqueous layer and transfer it to a new tube.
6. Sephacryl S-500 drip columns can be run "dry." A reservoir at the top of the column is not required. Each 150-µL wash yields an ~150-µL fraction volume. Fractions 1–3 can be collected in one tube since these fractions do not contain cDNA. The cDNA elutes in fractions 4 (containing fragments ≥1.5) and 5 (containing fragments >500 bp).
7. Ethidium bromide agarose plate quantitation is performed as follows. Using a DNA sample of known concentration (such as a plasmid), make serial dilutions (200, 150, 100, 75, 50, 25, and 10 ng/µL) in TE buffer. Melt 10 mL of 0.8% (w/v) agarose in TE buffer and cool to 50°C. Under a hood, add 10 µL of 10 mg/mL ethidium bromide, swirl to mix, and pour into a 100-mm Petri dish. Allow the plate to harden. Label the bottom of the Petri dish with a marker to indicate where the sample and standards will be spotted. Carefully spot 0.5 µL of each standard onto the surface of the plate. Do not puncture the agarose. Allow capillary action to pull the small volume from the pipet tip to the surface. Spot 0.5 µL of the cDNA sample onto the plate adjacent to the standards. Allow the spots to absorb into the agarose for 10–15 min at room temperature. Invert the plate and visual-