Primer Extension analysis of total yeast RNA

1. Mix the following in a 1.5 ml eppendorf tube:

a. 20-80 micrograms yeast RNA dissolved in H20 (the amount will depend on the level of expression for the gene of interest)

b. 2.0 microliters 5X annealing buffer

c. 200,000 - 400,000 cpm Kinased primer (typically about 1 ng)

d. H20 to a final volume of 10 microliters

2. Incubate tubes for 1 minute in a boiling water bath and then immediately transfer to 48 degree bath. Incubate for 45 minutes to allow annealing of primer and RNA.

3. Spin tubes briefly in a microfuge to bring down any condensation on the sides and top of tubes.

4. Add 20 microliters of Reverse Transcriptase Synthesis Mix. Incubate 30 min at 37 degrees.

5. Ethanol precipitate samples by adding 1/10 volume 3 M sodium acetate and 3 volumes ethanol. Freeze 10 min, spin, and wash pellets with 80% ethanol. Dry pellets in speedvac.

6. Resuspend pellets in 3.5 microliters 40 micrograms/ml RNAseA (this step degrades the yeast RNA which will interfere with the running of the sequencing gel). Incubate at room temp for 3 min.

7. Add 3.5 microliters Formamide sequencing gel loading buffer (a lab stock solution) and mix by vortexing.

8. Heat samples 90^{o} for 1 min and then chill rapidly on ice. Load samples to Urea/acrylamide sequencing gel.

Reverse Transcriptase Synthesis Mix:
4 microliters 5X synthesis buffer
0.3 microliters each dNTP (10 mM dATP, dCTP, dGTP, TTP)
14.3 microliters H2O
0.5 microliters M-MLV Reverse Transcriptase (200 units/microliter GIBCO/BRL)

5X Annealing Buffer	**1 ml**
25 mM Tris pH 8.3	25 microliters 1 M Tris 8.3
375 mM KCl	0.375 ml 1 M KCl
5 mM EDTA	20 microliters 0.25 M EDTA
580 microliters H2O	

5X Synthesis Buffer	**1 ml**
250 mM Tris pH 8.3	0.25 ml 1 M Tris 8.3
375 mM KCl	0.375 ml 1`M KCl
22.5 mM MgCl2	22.5 microliters MgCl2
75 mM DTT	150 microliters 0.5 M DTT
203 microliters H20	