

Primer Extension Analysis

- Prepare RNA from 2-5 OD₆₀₀ units of yeast collected at mid-log (OD₆₀₀ = 0.1-0.25). Do not resuspend RNA pellet in proteinase K + SDS. LiCl present in RNA samples must be removed, otherwise it will inhibit reverse transcriptase. Determine RNA concentration by measuring the A₂₆₀ of a diluted RNA sample (A₂₆₀ of 1 = 40 µg/ml). For 25S rRNA, 1 µg = ~1 pmol.
- Dilute oligonucleotide primer to a concentration of ~10 pmoles/µl.
- Prepare in vitro transcribed RNA control for concentration dependent pause (CDP) assay.
- For CDP assay, set up tubes "4", "20", and "100" in advance (see below).
- Partial alkaline hydrolysis. Use 2 µg of RNA (instead of 1 µg) for standard primer extension. Digest 2 µg RNA in 20 µl 50 mM Na₂CO₃, pH 9.0. 100 mM Na₂CO₃ = 1.06 g in 100 mls (check pH). Heat to 90°C for 3-5 minutes (try different times). Add KOAc, pH 5, and EtOH precipitate.

1. Set up reaction:	<u>Standard Primer Extension</u> 1 µl RNA (1 µg, ~1 pmol) 1 µl primer (10 pmol) 7 µl ddH ₂ O 1 µl "5X" Promega Buffer 10 µl Final Volume	<u>CDP Assay</u> 2 µl RNA (2 µg, ~2 pmol) 2 µl primer (20 pmol) 14 µl ddH ₂ O 2 µl "5X" Promega Buffer 20 µl Final Volume
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Set up reactions in 0.5 ml tubes. Use Promega buffer at "0.5X" (25 mM Tris-HCl, pH 8.3, 25 mM KCl, 5 mM MgCl₂, 5 mM DTT, 0.25 mM spermidine).

2. Denaturation: Incubate at 70°C for 10 minutes.
3. Annealing: Place at 42°C for 20 minutes.

4. Set up labeling:	<u>Standard Labeling Mix</u> 2 µl ddH ₂ O 5 µl 2 mM dCGT 1 µl [³⁵ S]dATP (10 µCi) 1 µl "5X" Promega Buffer 0.5 µl RNasin Promega, 10 U/µl <u>0.5 µl AMV RT</u> 10 µl Final Volume	<u>CDP Assay</u> — 5 µl 25 µM dCGT 2 µl [³⁵ S]dATP (20 µCi) 1 µl "5X" Promega Buffer 1 µl RNasin <u>1 µl AMV RT</u> 10 µl Final Volume
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See below for recipes for dCGT stock solutions.

For each RNA template prepare 10 µl of the labeling mixture.

Set up labeling mixtures on ice.

5. Add 10 µl of labeling mix. Incubate for 1 minute at 42°C.

6. Add dATP:	<u>Standard Labeling Mix</u> 1 µl of 10 mM dATP (to 20 µl)	<u>CDP Assay</u> 1 µl of 125 µM dATP (to 30 µl)
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7. Extension:	<u>Standard Labeling Mix</u> No additions	<u>CDP Assay</u> Transfer 10 µl to 3 tubes: "4", "20", "100"
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8. Incubate at 42°C for 10 minutes. Total volume for all samples is 20 µl at this step.
9. Place on ice. Add 25 µl of 10 mM EDTA.
10. Add 1 µl of 1 µg/µl heat-treated RNase A. Digest for 15 minutes at 37°C. Place on ice.
11. Add 5 µl (one-tenth volume) of 3 M NaOAc, pH 5. Mix completely.
12. Add 125 µl (2.5 volumes) of 100% ethanol. Mix completely. Place at -20°C for 1 hour to overnight.
13. Microcentrifuge for 30 minutes at 4°C.
14. Wash pellet at room temperature with 150 µl 70% ethanol for 5 minutes at 25°C. Spin for 5 minutes at 4°C.
15. Dry in Speed-Vac for about 5 minutes. It is not necessary to completely dry the pellet.
16. Resuspend pellets in 5 µl of Loading Buffer. Heat in steam bath 2 minutes, place on ice, load gel.
Loading Buffer: 50% formamide, 5 mM EDTA, 0.05% BPB (optional 0.05% XC).

dNTP Solutions

2 mM dCGT stock solution:	0.5 µl 100 mM dCTP 0.5 µl 100 mM dGTP 0.5 µl 100 mM dTTP 23.5 µl ddH ₂ O	Ultrapure 100 mM Stocks from Promega stored at -70°C Store at -70°C
25 µM dCGT stock solution:	dilute 2 mM dCGT 1/80.	

10 mM dATP stock solution:	dilute 100 mM dATP 1/10.	Store at -70°C
2 mM dATP stock solution:	dilute 10 mM dATP 1/5.	Store at -70°C
125 µM dATP stock solution:	dilute 2 mM dATP 1/16.	

Prepare "2X" Buffer by diluting 5X Promega Buffer 1 in 5. Final concentration will be 0.5X.

To prepare tube "100":

Make 400 µM dNTP stock. Mix 4 µl 2 mM dCGT + 4 µl 2 mM dATP + 12 µl ddH₂O.

Make 200 µM dNTP solution. Mix 10 µl of the 400 µM dNTP stock + 10 µl "2X" Promega Buffer.
Place 10 µl in tube labeled "100"

To prepare tube "20":

Make 80 µM dNTP stock. Mix 5 µl 400 µM dNTP + 20 µl ddH₂O.

Make 40 µM dNTP solution. Mix 10 µl of the 80 µM dNTP stock + 10 µl "2X" Promega Buffer.
Place 10 µl in tube labeled "20"

To prepare tube "4":

Make 8 µM dNTP stock. Mix 5 µl 80 µM dNTP + 45 µl ddH₂O.

Make 4 µM dNTP solution. Mix 10 µl of the 8 µM dNTP stock + 10 µl "2X" Promega Buffer.
Place 10 µl in tube labeled "4"