Northern Blot of tRNA in Yeast

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[Abstract] tRNAs are small RNAs around 70-90 nt. tRNAs are different from many other small RNAs in that they are very abundant, which makes it difficult to study their transcriptional regulation by traditional northern blot. Traditional northern blot involves incorporation of radioactive nucleotides through polymerization, however, tRNA is too short for polymerization. Traditional Northern blot detects changes in RNA levels, however, tRNA are so abundant that small changes in their levels will escape detection. For these reasons, metabolic labeling by radioactive uracil has been used instead. However, metabolic labeling can only examine changes in total tRNA, but cannot distinguish different types of tRNAs. The following protocol describes a method to examine individual tRNA gene transcription by northern blot.

Materials and Reagents

1. RapidGel (500 ml) (Affymetrix, catalog number: 75848)
2. Urea ($CO(NH_2)_2$) (Sigma-Aldrich, catalog number: U6504)
3. Tetramethylethylenediamine (TEMED, $C_6H_{16}N_2$) (Thermo Fisher Scientific, catalog number: 10-18-9)
4. APS/ Ammonium persulfate ($(NH_4)_2S_2O_8$) (Sigma-Aldrich, catalog number: A3678)
5. Formamide (CH_3NO) (Thermo Fisher Scientific, catalog number: 75-12-7)
6. DEPC/ Diethylpyrocarbonate ($O(COOC_2H_5)_2$) (Sigma-Aldrich, catalog number: D5758)
7. Bromophenol Blue ($C_{19}H_{10}Br_4O_5S$) (Sigma-Aldrich, catalog number: B0126)
8. Xylene Cyanol FF ($C_{25}H_{27}N_2NaO_6S_2$) (Sigma-Aldrich, catalog number: X4126)
9. Probe DNA oligo
10. [γ-^{32}P]-ATP, 10 Ci/mmol 2 mCi/ml, 250 µCi (PerkinElmer, catalog number: BLU002250UC)
11. Whatman 3 M paper (Whatman, catalog number: 3 MM Chr)
12. Microspin G-25 column (GE Healthcare, catalog number: 27-5325-01)
13. Hybond N+ membrane (Amersham, catalog number: RPN303B)
14. Ficoll (Sigma-Aldrich, catalog number: F4375)
15. Sodium citrate
16. BSA

17. Polyvinlypyrrolidone (Sigma-Aldrich, catalog number: PVP360)
18. T4 Polynucleotide Kinase (New England Biolabs, catalog number: M0201S)
19. 10x PNK buffer (New England Biolabs, catalog number: B0201S)
20. Tris base (Thermo Fisher Scientific, catalog number: 77-86-1)
21. Boric acid (Thermo Fisher Scientific, catalog number: 10043-35-3)
22. EDTA (Sigma-Aldrich, catalog number: EDS-1KG)
23. Methylene blue (Sigma-Aldrich, catalog number: 28514-100G)
24. Sodium acetate (Sigma-Aldrich, catalog number: S2889)
25. Salmon sperm DNA (Life Technologies, Invitrogen™, catalog number: 15632-011)

Equipment

1. Bench top centrifuge
2. UV cross linker (Strata linker, model: 1800)
3. Power supply with constant voltage > 450 V
4. Exposure cassette / intensifier screen (Sigma-Aldrich, catalog number: C5479-1EA)
5. Hybridization bottle
6. Hybridization oven
7. Large gel cassette (around 20 x 40 cm)
8. Film developer

Procedure

Part I: Separate tRNAs by denaturing gel

1. Prepare total RNA by either Trizol RNA extraction kit or by hot phenol method described in Wei (2012). Good quality RNA will have an $OD_{260/280}$ ratio of 1.8 to 2 and an $OD_{260/230}$ of 1.8 or greater.
2. Prepare 10% Acrylamide-6 M Urea denaturing gel in a large gel cassette (around 20 x 30 cm. Mini gel did not work well in my experiment).
3. Pre-run the gel in 1x TBE buffer with constant 450 V until the gel is heated to 50 °C (about 1 h). *Note: I found this to be critical. One reason could be that pre-running the gel to this temperature could help get rid of excessive Urea in the gel, making RNA possible to go through. I usually attached a thermometer to ensure that the temperature reach 50 °C.*
4. RNA samples are mixed with 2x with Formamide loading dye and heated to 70 °C for 2 min.

5. Turn off power. Rinse out the wells with 1x TBE using a syringe and needle, make sure that urea is rinsed out.
6. Load the RNA samples (25 µg) and run the gel at constant 450 V for about 2 h (Bromophenol Blue runs around 12 nt and cyanol around 55 nt).
7. Let the gel cool down to room temperature and disassemble the gel set.

Part II: Transfer tRNAs to membrane

8. Measure gel and cut a piece of Hybond N^+ membrane with clean gloves and scissors (washed with DEPC water and autoclaved). Soak the membrane in 1x TBE, attach to the gel. Soak 2 pieces of autoclaved Whatman 3 M paper in 1x TBE and sandwich the gel-membrane.
9. Transfer gel/membrane/whaman paper sandwich to semi-dry transfer apparatus. Use a sterile transfer pipet to squeeze out bubbles between the membrane and the gel.
10. Wipe off excess liquid around the edges of gel and transfer at constant 10 V for 2.5 h.
11. Disassemble the gel and membrane, soak membrane in DEPC water for 1 min. Put the membrane on autoclaved filter paper for 10 min to dry the membrane.
12. Crosslink the RNAs to the membrane with UV cross linker (Strata Linker 1800) at 1,200 x 100 m Joles. It takes roughly 2 min.
13. Stain with methylene blue RNA staining solution for 1 min. De-stain by washing with DEPC water several times for 30 sec. This is to evaluate the efficiency of RNA transfer. If transfer is not good, I suggest repeating the above procedures since radioactive material is involved in later steps.
14. Save the membrane in DEPC water for Northern blotting.

Part III: 5’ End-Labeling of Probe

15. To examine the newly synthesized tRNA, design DNA oligo annealing specifically to the pre-tRNA rather than mature tRNA, because mature RNA is very abundant. Pre-tRNAs will be processed to mature tRNA. During this process, certain DNA sequence will be cleaved. Primers will be designed annealing to the cleaved DNA sequence For example, pre- tRNALeu3 probe is 5'-CCAAACAACCACTTATTTGTTGA-3'.
16. Prepare 5’ end labeling reaction buffer and label the probe with radioactive ATP at 37 °C for 1 h (see recipes for detail).
17. Prepare the microspin G-25 column by vortexing the resin in the column.
18. Snap off bottom and loosen the cap, place in 1.5 ml Eppendorf tubes and spin the column for 1 min at 700 *x g*. The gentle centrifugation is critical for the column to form a smooth angled surface.

19. Place the column in new Eppendorf tube and slowly apply the labeling reaction to center of angled surface of resin bed. Do not disturb the resin.
20. Spin column for 2 min at 700 *x g*. Discard column in radioactive waste.
21. Take out 1 µl of labeled probe in 0.5 ml Eppendorf tube, put tube in scintillation vial and count the cpm.

Part IV: Hybridization

22. Mix hybridization buffer and take 20 ml into 50 ml conical tube.
23. Heat the buffer in 65 °C water bath until clear. It should be clear within 10 min.
24. Transfer 10 ml to hybridization bottle with membrane (note transferred side is the proved side) and rotate gently at 37 °C in a hybridization oven for 60 min.
25. Put the remaining 10 ml hybridization at 37 °C for 30 min.
26. Heat 100 µl salmon sperm DNA at 90 °C for 1 min and add to the remaining 10 ml hybridization buffer.
27. Add 20 µl labeled probe into the 10 ml hybridization buffer and shake vigorously.
28. Decant the buffer in the hybridization bottle and add the new hybridization buffer with labeled probe. Rotate gently at 37 °C in a hybridization oven overnight.
29. Pre-warm wash buffer to 37 °C in a water bath.
30. Transfer hybridization buffer with probes to 50 ml conical tube and store it at -20 °C. The probes can be re-used within 2 weeks for 2-3 times, but the auto-radioactive signal will be decreasing.
31. Wash the membrane with 50 ml wash buffer by rotating at 42 °C for 30 min in the hybridization oven.
32. Repeat washing for 2 more times.
33. Take out membrane and wrap with Saran-wrap.
34. Place the membrane in exposure cassette with intensifier screen and film, put at -80°C refrigerator.
35. Develop the film after 3 days. If signal is weak, expose for 1 week.

Recipes

1. DEPC water
   Add 1 ml DEPC to 1 L ddH_2O_2, mix and put at room temperature overnight.
   Autoclave.
2. 10x TBE in DEPC water
   In 800 ml DEPC water, add 108 g Tris base, 55 g boric acid, 40 ml of 0.5 M EDTA (pH 8.0).

Mix to dissolve and add DEPC water to 1 L.

Autoclave.

3. 10% Acrylamide-6 M Urea denaturing gel
   a. Mix 2.5 ml 10x TBE, 6.25 ml Rapid Gel (40%) and 15 g Urea, heat to 50 °C and mix to dissolve.
   b. Add DEPC water to 25 ml then filter through 0.45 μM filter syringe.
   c. Add 25 μl TEMED and 50 μl 25% APS, mix vigorously and transfer to large gel set with appropriate comb.
4. 2x Formamide loading dye

   95% (v/v) formamide in DEPC water, add tiny amount of Bromophenol Blue (0.01~0.1%) and Xylene Cyanol FF (0.01~0.1%), vortex to mix.
5. Methylene blue RNA staining solution

   Dissolve sodium acetate in water to 0.3 M and adjust to pH 5.5 with Glacial Acetic acid and NaOH. Dissolve 0.02 g methylene blue in 100 ml of above solution and filtered.
6. 5' end labeling reaction buffer

   2 μl 10x PNK buffer (fresh from NEB)

   30 pmol probe DNA oligo

   3.3 μl [γ-^{32}P] ATP

   1μl T4 Polynucleotide Kinase, 10 U/ml

   Add DEPC H_2O to 20 μl

   Incubate at 37 °C at least 1 h.
7. 20X SSC (1 L)

   NaCl 175 g

   Sodium citrate 88 g

   Add DEPC water to 1 L and autoclave
8. 50x Denhardt's solution (100 ml)

   Ficoll 1 g

   Polyvinlypyrrolidone 1 g

   BSA 1 g

   Add DEPC water to 100 ml to dissolve, filter solution through 0.45 μl filter syringe.
9. Hybridization buffer (1 L)

   250ml 20x SSC

   100ml Denhart's reagent (50x)

   10 ml 10%SDS (autoclaved)

   Add DEPC water to 1 L, store in 4 °C
10. Wash buffer (1 L)

    100 ml 20x SSC

10 ml 10%SDS (autoclaved)

Add 890 ml DEPC water and store at room temperature.

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References

1. Wei, Y., Tsang, C. K. and Zheng, X. F. (2009a). Mechanisms of regulation of RNA polymerase III-dependent transcription by TORC1. *EMBO J* 28 (15): 2220-2230.
2. Wei, Y. and Zheng, X. F. (2009b). Sch9 partially mediates TORC1 signaling to control ribosomal RNA synthesis. *Cell Cycle* 8(24): 4085-4090.
3. Wei, Y. (2012). A simple preparation of RNA from yeast by hot phenol for northern blot. *Bio-protocol* 2(9): e209.