tRNA Northern Analysis

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Culture Growth

1. Grow an overnight 5.0 ml LB culture of the strains to be evaluated.
2. Inoculate the overnights to 30 ml LB and do a short growth curve to get the cells at mid log-phase (OD_{600} = 0.5).
3. Pellet cells at 5k, 5 min., 4°C. All steps and solutions from here forward are to be on ice.
4. Re-suspend pellet in 300μl cold Sodium Acetate (0.3M, pH 4.5), 10 mM EDTA.
5. Cells added to 0.3 g of glass beads and snap froze in etoH and dry ice bath then transferred to –20 degrees C. hold samples here until all cultures are at this point

tRNA Extraction

6. Add 300μl phenol:chloroform (pH 4.7) to the samples.
7. Vortex eppy 4-5 times for 30 second bursts with incubation on ice in between.
8. Centrifuge samples for 15 minutes, 4°C, max speed.
9. Transfer top layer (aqueous phase) to new tubes and repeat the phenol extraction by adding 300ul phenol:chloroform (pH 4.7), spinning for 15 minutes, 4°C, max speed & removing the aqueous phase to a fresh eppy.
10. The RNA is etoH precipitated by adding 3 volumes of cold 100% etoH and spinning at max speed for 25 minutes, 4°C.
11. RNA pellet is re-suspended in 60 ul cold sodium acetate (0.3 M, pH 4.5).
12. Precipitate the RNA by adding 400 ul 100% etoH and spinning at max speed for 25 min., 4°C. decant.. spin again 1 min. and remove traces of etoH w/ pipette..
13. air dry the pellet while on ice..
14. re-suspend the pellet in 50 ul cold sodium acetate (10 mM, pH4.5).
15. quantitate RNA by spectrophotometry (A_{260}) and analyze the integrity of the RNA by running a 2.0% agarose gel.

Gel Electrophoresis

16. Mix the RNA sample with 2X loading buffer. (recipe step #18)

17. Run the14% denaturing acid gel at 50 volts, 4°C for 24 hours ..

    Using BioRad miniprotein II gel rigs (1.0 mm)…. make ea. gel as follows..
    3.5 ml - 40% polyacrylamide (19:1)
    4.2 g - urea
    3.0 ml – sodium acetate 1.0 M (pH 5.0)…….. final concentration is 0.3M
    melt this urea by heating in 70 degree C H2O bath

pH adjust the melted acrylamide/urea gel to pH 5.0 by adding acetic acid as needed (spotting onto pH paper) de-gass gel solution and polymerize w / 34 ul TEMED & 124 ul 10% amonium persulfate.

18. Make the 2X loading dye..

    4.2 g - urea

    3.0 ml – sodium acetate 1.0 M (pH 5.0)…….. final concentration is 0.3M

    melt the urea by heating in 70 degree C H2O bath, Qs volume to 10.0 ml w/ H2O

    pH adjust the melted solution to pH 5.0 by adding acetic acid as needed

    add 5 ug xylene cyanol and 5 ug bromophenol blue

19. tRNA de-acylation: add an equal volume of (100 mM Tris-Hcl, 100 mM NaCl pH 9.5)
    to RNA samples and incubate 70 degree C, 30 min.

20. electrophoresis buffer: 0.3 M sodium acetate pH5.0

IMPORTANT NOTE:

The pH of the buffer will drift during the electrophoresis run… the inside will become very basic and this will cause the de-acylation of samples… 0.3 M sodium acetate pH 5.0 will be stable for 7-8 hours of electrophoresis.. the buffer must be routinely removed from the unit and mixed, then returned to continue the run. for the entire 24 hour run, 50V, 4°C. The tRNAs should have migrated to about ¾ down the gel. The 0.3 M sodium acetate electrophoresis buffer must be pH adjusted using acetic acid and not HCl.

Transfer

20. Electroblot the RNA to nylon membranes using the biorad western blotting
    sandwhich cassettes… transfer buffer:

final concentration	1 liter
Tris-Oac pH 7.8 (10 mM)	10 ml 1.0 M Tris-Oac pH 7.8
Sodium Acetate (5 mM)	0.41 g Sodium Acetate
EDTA (0.5 mM)	1.0 ml EDTA (0.5 M pH 8.0)

Transfer conditions: 40v, 2 hours, 4 degree C.

21. UV crosslink RNA to membrane using optimal setting on UV crosslinker. After crosslinking any blocking/hybridization/detection method of your choice can be applied to the blot (see note at end of protocol about detection).

Hybridization & Detection

22. DIG tailed probe prepared per manufacturer's protocol.
23. Prehybridize membrane 2 hours 42 degrees C in hyb bottles w/ 25 ml prehyb.

    50 ml prehyb buffer

    500 ul -- 10% N- lauroylsarcosine

    12.5 ml ---20 x SSC

    5 ml -------10 X blocking reagent (Roche cat # 1096176)

    50 ul ------20% SDS

    300 ul ----salmon sperm DNA (3.7 mg/ml). QS to final volume of 50 ml w/ ddH2O
24. Hybridize w/ 1.5 pmol DIG tailed probe / ml hyb solution Overnight 42 degrees C

    50 ml hyb buffer

    500 ul -- 10% N- lauroylsarcosine

    12.5 ml ---20 x SSC

    5 ml -------10 X blocking reagent (Roche cat # 1096176 diluted in maleic acid buffer)

    50 ul ------20% SDS

    QS to final volume of 50 ml w/ ddH2O
25. Post-hybridization washes

    1 wash for 15 min, 42 degrees C, (6XSSC, 0.1%SDS)

    2 washes for 15 min. ea., 42 degrees C, (4XSSC, 0.1%SDS)

    1 wash for 15 min, room temp, (2XSSC, 0.1%SDS)
26. Dig detection:

- equilibrate filter for 2 min. in detection buffer (100 mM Tris-Hcl, 100 mM NaCl pH 9.5)
- block filter 30 min. in 1X blocking reagent (the 10 X blocking reagent diluted in maleic acid buffer) room temperature.
- hybridize anti-DIG AP FAB fragments (1:10,000) in 1X blocking reagent for 30 min room temp.
- wash filters 2 X in washing buffer (maleic acid buffer w/ 0.3% Tween)
- dropwise add substrate (CSPD ready to use) onto filters.. seal in bag.
- incubate filters in the bag at 37 degree C for 15 min..

then expose filters to film for a duration of time to give a good exposure, picture.

Several things to consider about detection!

This protocol outlines the use of DIG-tailed oligos. From experience… I have found that the addition of the DIG-UTPs to the end of the oligo can cause hybridization problems in some circumstances. For hybridization/detection I have had much more reproducible results when I switched to using biotinylated oligos and the Phototope®-Star Detection Kit from NEB. Obviously, using radioactive probes would also cause no hybridization interference and works quite well.

Best of luck!

If using this protocol, please cite original publication:

Jester BC, Levengood JD, Roy H, Ibba M, Devine KM. Nonorthologous replacement of lysyl-tRNA synthetase prevents addition of lysine analogues to the genetic code. *Proc Natl Acad Sci U S A.* 2003 Nov 25;100(24):14351-6