tRNA Northern Analysis

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Abstract

This protocol provides a means to define the level of aminoacylation for a specific tRNA in vivo.

Introduction

Herein describes a method for extracting total RNA from a cell, resolving the charged and uncharged species by electrophoresis and the blotting/detection of a specific tRNA species.

Reagents

LB media

Sodium Acetate 0.3M (pH 4.5), 10 mM EDTA.

sodium acetate 10 mM (pH4.5)

sodium acetate 1.0 M (pH 5.0)

sodium acetate 0.3 M (pH5.0)

etoH

phenol:chloroform (pH 4.7)

40% polyacrylamide (19:1)

urea

100 mM Tris-Hcl, 100 mM NaCl pH 9.5

1.0 M Tris-Oac pH 7.8

EDTA (0.5 M pH 8.0)

Equipment

incubator to grow culture

centrifuge

microfuge

vortex

spectrophotometer

agarose gel electrophoresis

BioRad miniprotein II rig for gel running/blotting

hot water bath

UV crosslinker

hybridization oven

Procedure

Culture Growth (*B. subtilis*)

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 (OD600 = 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 300ml 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 300ml 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 (A260) 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. Repeat for the entire 24 hour run, 50V, 4∞C. The tRNAs should have migrated to about 3/4 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

Hybridization & Detection (using DIG tailed oligos)

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.

Timing

To grow cells, an overnight preculture followed by a 2-3 hour culture to obtain logarithmic growing cells.

Isolation of total RNA and preparation/loading of the gel takes approximately 4-5 hours.

the electrophoresis run is 24 hours.

blotting and blocking takes 4 hours

hybridize overnight

washes and detection takes about 2 hours

Total Time: 3 days

Troubleshooting

Several things to consider about detection!

This protocol outlines the use of DIG-tailed oligos. From experience... We have found that the addition of the D

Anticipated Results

You should have two bands resolved on your blot. The upper band is the aminoacylated tRNA and the bottom band is the uncharged species. The lane that contains the deacylated sample will have only the lower band which corresponds to the tRNA only.

References

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

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

tRNANorthern.pdf

Stationary-phase expression and aminoacylation of a transfer-RNA-like small RNA

by Sandro F Ataide, Brian C Jester, Kevin M Devine, +1

EMBO reports (26 April, 2011)

The T box regulatory element controlling expression of the class I lysyl-tRNA synthetase of Bacillus cereus strain 14579 is functional and can be partially induced by reduced charging of asparaginyl-tRNAAsn

by Niall Foy, Brian Jester, Gavin C Conant, +1

BMC Microbiology (26 April, 2011)

A natural genetic code expansion cassette enables transmissible biosynthesis and genetic encoding of pyrrolysine

by D. G. Longstaff, R. C. Larue, J. E. Faust, +4

Proceedings Of The National Academy Of Sciences (26 April, 2011)