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VISUAL EXPERIMENT

Detection of cytosolic tRNA in mammal by Northern blot analysis

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ABSTRACT

Out of entire cascade of technologies and strategies, Northern blot assay remains the most preferential approach for immediate and accurate evaluation of expressed RNA species. However, an abundance of tRNAs species under physiological conditions compared to other small RNAs makes it difficult to accurately evaluate their transcriptional alterations through traditional Northern blot assay. Here, we describe an efficient protocol for detecting subtle alterations in tRNA species in mammals by a modified Northern blot assay. This report also compares the chemical versus UV-based crosslinking of tRNA species to the surface of solid supports.

INTRODUCTION

Transfer RNAs (tRNAs) are small adapter molecules of approximately 70 nucleotides in length that translate codons in mRNA into their cognate amino acids in a polypeptide chain. Protein biosynthesis process requires tRNA molecules to actively participate in interaction with several proteins such as tRNA modifying enzymes, aminoacyl-tRNA synthetases, translation initiation and elongation factors, and a repertoire of ribosomal RNAs, proteins and mRNA, therefore, estimation of alterations in tRNA profile has become quintessential. Last decade has fuelled the development of powerful technologies dedicated for profiling of steady state accumulation of specific small RNA species.

Northern blot assay was developed in the year 1977 (Alwine et al., 1997) and since then, it has revolutionized the RNA biochemistry field. Past decades have experienced spectacular progress in the molecular biology through the advent of powerful technologies developed dedicated for quantifying and manipulating RNA species expressed in the cell (Gesteland et al., 2006). However, Northern blot forms the basic fundamental, quick and clean, easy-to-go technique behind more sophisticated and costly RNA-based analysis. The tRNA Northern blot procedure has been adapted from Wei et al., (2013).

MATERIALS AND EQUIPMENTS

1. Hoefer™ SE 600 Series vertical electrophoresis unit (Hoefer™ SE600, Catalog No. 03-500-238)
2. Power supply with constant voltage >450 (GE Healthcare electrophoresis power supply- EPS601)
3. Sterilized micropipette and pipette tips
4. Digital gel imaging system
5. Bench top centrifuge
6. Semi-dry transfer cell (TANON VE- 386)
7. UV crosslinker (VUP CL-1000 ultraviolet crosslinker)
8. Hybridization bottles
9. Hybridization oven (VUP HB-1000 Hybridizer)
10. Temperature regulated bench top water bath

11. Whatman 3M blotting paper (Whatman, Catalog No. 3 MM Chr)
12. Purification kit, Illustra Microspin G-25 column (GE Healthcare, Catalog No. 27-5325-01)
13. Hybond N+ membrane (Amersham, Catalog No. RPN303B)
14. Neutral Nylon membrane, Hybond NX (GE Healthcare Life Science, Catalog No. RPN203T)
15. Geiger Mueller Detectors
16. Exposure cassette and intensifier screen (GE Healthcare)

REAGENTS AND SOLUTIONS

- Urea (ultra-pure, Sigma-Aldrich, Catalog No. U6504)
- 40% Polyacrylamide solution (Bio-Rad, 29:1 3.3% crosslinker, Catalog No. 161-0146)
- N,N,N′,N′-Tetramethylethylenediamine (TEMED, $C_6H_{16}N_2$) (Sigma-Aldrich, Catalog No. T9281)
- 10 % Ammonium persulfate (APS, $(NH_4)_2S_2O_8$) (Sigma-Aldrich, Catalog No. A3678)
- Formamide (Bio Basic Canada Inc. CAS-75-12-7)
- Diethylpyrocarbonate (DEPC, $O(COOC_2H_5)_2$) (Bio World, SKU: 40400024-3)
- Xylene Cyanol FF ($C_{25}H_{27}N_2NaO_6S_2$) (Sigma-Aldrich, Catalog Number: x-4126)
- Bromophenol Blue ($C_9H_{10}Br_4O_5S$) (Sigma-Aldrich, Catalog No. 114391)
- Tris Base (Bio Basic Canada, CAS-77-86-1)
- Boric Acid (Bio Basic Canada, CAS-10043-35-3)
- EDTA (Daejung, Catalog No. 60-00-4)
- DNA probe oligonucleotides
- Mammalian cytosolic methionine initiator tRNA (TRX-CAT1-1 gene) probe sequence CGATCCATCGACCTCTGGGTTATGGGCCCAGCACGCTTCCGCTGCGCCACTCTGCT
- Mammalian cytosolic tRNA for glycine (GCC-anticodon) probe sequence GCATTGGCCAGGAATCGAAGCCCGG
- ATP, [γ-^{32}P]- 3000 Ci/mmol 10 mCi/mL, 100 µCi (PerkinElmer, Catalog No. 5000051)
- T4 Polynucleotide kinase (Enzynomics, 10 U/µL, Catalog No. M0055)
- T4 Polynucleotide kinase buffer
- Ficol 400 (Sigma-Aldrich, Catalog No. F2637)
- Bovine serum albumin (BSA, Sigma-Aldrich, Catalog No. A7906)
- Polyvinylpyrrolidone (Sigma-Aldrich, Catalog No. PVP40)
- Methylimidazole (Sigma-Aldrich, Catalog No. 336092)
- HCl (Sigma-Aldrich, Catalog No. H1758)
- Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma-Aldrich, Catalog No. 3450)
- Salmon sperm DNA (0.1 mg/mL) (Sigma-Aldrich, Catalog No. D1626)
- Sodium citrate tribasic dehydrate (Sigma-Aldrich, Catalog No. S4641)
- Sodium chloride
- RNase Wiper (iNtRonBuiotechnology, Catalog No. 21131)

VIDEO CLIP

Duration: 16 min 54 sec

PROTOCOL

The protocol takes three days to complete and further two to three days to expose and develop the autoradiography film.

Denaturing urea polyacrylamide gel electrophoresis

1. Perform total RNA extraction from desired source (tissue, monolayer cells, suspension cells) using commercial reagents e.g., TRIZOL reagent RNA extraction protocol by following manufacturer's instructions and recommendations
2. In order to assess the quality of RNA load 2 µg of total RNA on 0.8% EtBr-agarose gel and run the horizantal electrophoretic unit at 50 V in 1x TBE buffer until RNA species are resolved (15 ~20 min). Identify the distinct bands corresponding to 28S, 18S, and small RNA species such as 5.8S and tRNAs
3. For the RNA fractionation step, use large vertical electrophoretic apparatus (Plate size 20 cm x 16 cm, comb size 1 mm). Proceed with the assembly of clean glass plates together in casting frame by following manufacturer's description. Larger gels are used in order to enhance resolution and separate bands with a difference of single nucleotide
4. Prepare 60 mL of 15 % acrylamide/bisacrylamide (29:1) casting solution in 1x TBE, 8M ultra pure urea and fill the volume by DEPC treated deionized water. Dissolve the components by swirling the flask and sterilize the gel casting solution by passing through a 0.4 µM filter with the help of 50 mL syringe. Immediately, before pouring add 590 µL of 10 % APS and 24 µL of TEMED. Pour the gel immediately in between the vertical plates in gel casting frame and avoid introduction of air bubbles. Carefully, insert the comb and allow the gel to polymerize for 1 hour at room temperature before use

RNA sample preparation

1. Aliquot 20-30 µg of total RNA for each sample and add an equal volume of 2x acrylamide blue loading dye (2x LD)
2. Denature the RNA sample by heating at 80^{o}C for 10 min and chill on ice until loading

Fractionation by electrophoresis

1. Gently remove the comb and dismount the gel from casting frame. Carefully assemble the gel in the electrophoretic cell, following manufacturer's instructions
2. Fill the lower and upper chamber with 1x TBE running buffer. The lower and upper chamber should be filled with running buffer to the same level
3. Attach the lid and connect the cables to the high voltage power supply. Apply the constant voltage ~450 V and pre-run the electrophoretic unit for 1 hour
4. Rinse the well pockets by squirting 1x TBE buffer through a sterile syringe
5. Carefully load samples from the bottom of the well. Assemble the apparatus and apply constant voltage ~100 V for 30 min, followed by an increase in voltage to ~200 V and run for 4-6 hours
6. When the bromophenol blue dye front reached the end of the gel, carefully dismantle the assembly and de-clamp the gel. Cut the gel into pieces as per your requirement
7. In order to visualize fractionated RNA on the gel, incubate the gel slice in 1x TBE buffer containing sufficient ethyl bromide, at room temperature for 20 min and place on the slow rocker. Additional RNA sample should be loaded as a control for fractionation on gel. Destain the gel in 1x TBE for 20 min and visualize fractionated RNA by UV irradiation based gel imaging system

Gel blotting

1. Measure the gel and carefully cut 6 pieces of Whatman blotting paper and nylon membrane of equivalent size. Equilibrate the gel pieces, blotting papers and nylon membrane in 1x TBE

2. Clean the surface of semi-dry transfer apparatus with RNase Wiper and place 3 pieces of soaked blotting paper on the surface of apparatus. Carefully remove air bubbles and excess TBE buffer by means of a sterile roller and dab the surface from the edges
3. Place the buffer-equilibrated nylon membrane on the blotting papers and remove the excess TBE buffer from the edges. Place the gel containing fractionated RNA on the nylon membrane and remove the excess TBE buffer
4. Sandwich the nylon membrane and gel by covering with more 3 soaked blotting papers and carefully roll out the air bubbles and excess TBE buffer using a roller
5. Carefully check the orientation of electrodes and assemble the transfer apparatus
6. Transfer RNA from the gel to the nylon membrane at constant 10 V for 2.5 hours

The schematic of Northern blotting is shown in Figure 1.

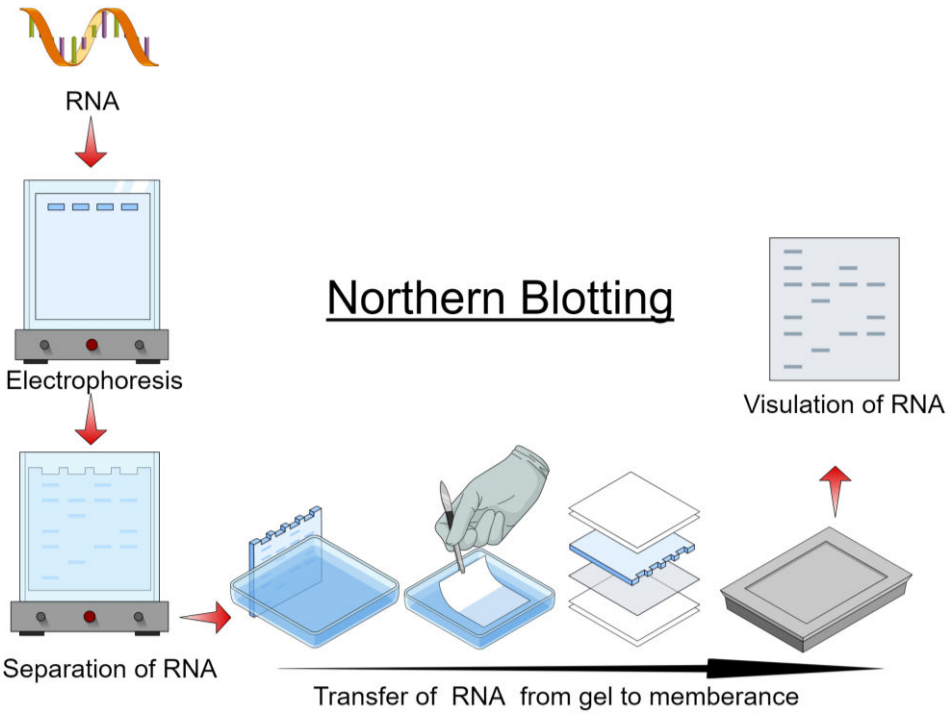

Figure 1: General schematic of Northern blotting

CROSSLINKING

UV RNA crosslinking

1. Dismantle the transfer apparatus and soak the procured membrane in DEPC-treated water
2. Place the membrane on an autoclaved sterile blotting paper with RNA side facing up and allow it to dry for 10 min
3. Place the membrane in UV cross linker chamber and expose to UV at 1200 x 100 mJ for almost 2-3 min

Chemical RNA crosslinking

1. Reconstitute required volume (10 mL) of the fresh crosslinking solution. Equilibrate a sterile piece of blotting paper with crosslinking solution by placing on a clean glass plate
2. Dismantle the transfer apparatus and place the membrane on soaked blotting paper, with RNA side facing up and cover the surface with another clean glass plate
3. Wrap the sandwich in saran wrap and heat the assembly at 60°C for 2 hours

4. Post crosslinking place the membrane in DEPC-treated distilled water

Membrane Pre-hybridization

1. Pre-warm hybridization solution at 37°C and heat denature salmon sperm DNA at 95°C for 5 min, snap chill on ice
2. Add 100 µL of denatured salmon sperm DNA to 10 mL of hybridization solution in hybridization bottle and place the membrane with RNA side facing the buffer
3. Incubate the membrane at 37°C for 1 hour under constant rotation in hybridization oven

5' End labelling of DNA probe

1. Prepare the phosphate forward kinase reaction and radiolabel the probe at 37°C for 1 hour
2. Purify the labeled probe from unincorporated radioactive nucleotides by gel exclusion chromatography on microspin G-25 column following the manufacturer's instructions
3. Briefly, vortex the resin in the column. Click snap the bottom of the column, place it in 1.5 mL Eppendorf tube, slightly loosen its cap and centrifuge at 700 x g for 1 min
4. Place the column in fresh 1.5 mL Eppendorf tube without disturbing the smooth angled surface of the resin. Carefully apply the labeling mixture at the center of the inclined bed and centrifuge for 2 min at 700 x g
5. Discard the column in radioactive waste and assess the probe activity by liquid scintillation counter

Hybridization

1. Replace the pre-hybridization solution from the bottle with 10 mL of fresh warm hybridization buffer
2. Heat the labeled probe at 95°C for 10 min, snap chill on ice and add to fresh hybridization buffer
3. Incubate the membrane at 37°C overnight in rotating assembly of hybridization oven

Membrane washing and signal detection

1. Replace the hybridization buffer with 50 mL of wash buffer
2. Incubate the membrane in wash buffer at 42°C for 30 min under maximum rotation in the hybridization chamber
3. Repeat the washing step 2 more times
4. Use Geiger-Muller counter for detecting residual radioactivity at the membrane edges. With background emission values of around 5-10 cps, proceed ahead for setting up membrane exposure assembly
5. Assemble the membrane/film sandwich using exposure cassette and intensifier screen following manufacturer's recommendations in dark room and allow the film to expose for almost 2 days and develop the exposed film using film developer protocol

Preparation of solutions

1. DEPC-treated distilled water (1 L)

   Add 1 mL DEPC to 1 L distilled water, shake well and incubate at room temperature overnight, followed by autoclaving at 121°C for 30 min

2. 10x Tris-borate EDTA (TBE) running buffer (1 L)

Tris-base	108 g
Boric acid	55 g

0.5 M EDTA	40 mL
DEPC-treated water	700 mL

Mix the components well and fix pH to 8.0. Add DEPC-treated water to make final volume up to 1 L and autoclave

3. 15% Acrylamide/bisacrylamide-8 M urea denaturing gel (60 mL)

40% Acrylamide/bisacrylamide (29:1)	22.5 mL
10x TBE	6 mL
8 M Urea	28.5 g

Mix the components by swirling in the flask and make the final volume up to 60 mL by adding DEPC-treated water. Sterilize by passing through 0.4 µM filter syringe

Add 24 µL of TEMED and 590 µL of 10% APS, mix vigorously and pour immediately into the vertical casting frame. Carefully, fix appropriate comb and allow the gel to solidify for 1 hour

4. RNA Loading dye (2x LD) (1 mL)

10x TBE	100 µL
Formamide	900 µL
Xylene cyanol	1 mg
Bromophenol blue	1 mg

Mix by vortexing

5. 5′ End labeling reaction (20 µL)

10x PNK buffer	2 µL
DNA oligo probe	30 pmol
[γ-^{32}P] ATP	4 µL (>1000000 cpm)
T4 Polynucleotide kinase	1 µL
DEPC-treated water	10 µL

Mix the components and set up the kinase forward reaction at 37^{o}C for 1 hour. Separate the labeled probe from free nucleotides according to the instructions mentioned in the protocol

6. Crosslinking solution (10 mL)

1% 1-methylimidazole (v/v)	100 µL
12.5 mM HCl	10 µL
3.1% EDC	310 mg
DEPC-treated water	9890 µL

Mix the components just before use

7. 50x Denhardt's solution (100 mL)

Ficoll (1%)	1 g
Polyvinyl pyrolidone (1%)	1 g
BSA (1%)	1 g

Add DEPC-treated water to make final volume up to 100 mL and vacuum filter sterilize through 0.4 µM filter

8. 20x, Sodium chloride sodium citrate (SSC) buffer (1 L)

3 M NaCl	175 g
300 mM Tri sodium citrate	88 g

Add DEPC-treated water to make final volume up to 1 L and autoclave

9. Hybridization solution (1 L)

20x SSC buffer	250 mL (5x final concentration)
50x Denhardt's solution	100 mL (5x final concentration)
10% SDS	10 mL (Filter sterilized, 0.1% final concentration)

Add DEPC-treated water to make final volume up to 1 L and store at 4°C

10. Wash buffer

20x SSC buffer	100 mL (2x final concentration)
10% SDS	10 mL (Filter sterilized, 0.1% final concentration)

Add DEPC-treated water to make final volume up to 1 L and store room temperature

RESULTS

We followed the procedure described in the text and estimated the cytosolic methionine initiator tRNA and cytosolic glycine tRNA expression from samples of mammalian origin. We also compared the tRNA crosslinking efficiency to a solid support using UV crosslinker strategy and carbodiimide based chemical cross linking technology. RNA was isolated from HEK293T monolayer cells by using TRIZOL reagent method and integrity of RNA was assessed by visualizing discrete bands on an agarose gel and also by estimating $OD_{260/280}$ (~2) and $OD_{260/230}$ (~1.9) ratio on NanoDrop.

RNA samples (30 µg) for cytosolic methionine initiator tRNA were run in duplicates. One replicate was blotted on neutral nylon membrane and chemically cross linked by EDC whereas, another replicate was blotted on negatively charged nylon membrane and crossed by UV.

The expression of cytosolic methionine initiator tRNA was detected and confirmed through both methods (Figure 2). However, the chemical crosslinking with EDC exhibits higher sensitivity and enhanced specificity than traditional UV crosslinking. Therefore, utilization of EDE based chemical crosslinking could largely benefit small sized and less abundant species of tRNA molecules.

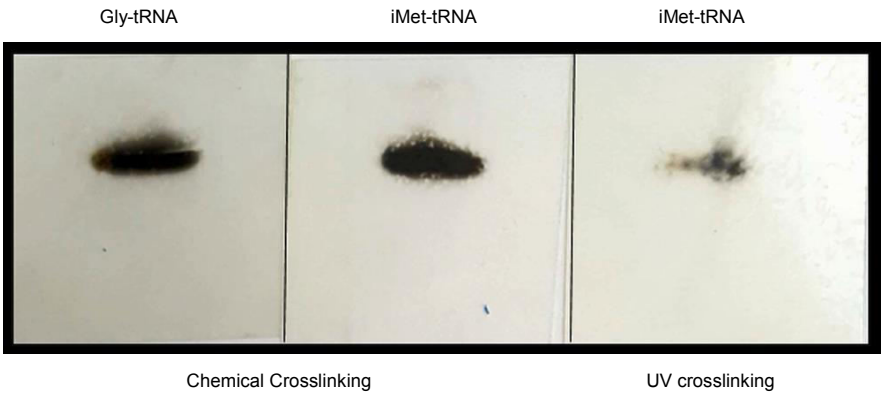

Figure 2: Northern blot for detection of methionine initiator tRNA. Equal amounts (30 µg) of RNA sample was resolved on a denaturing urea polyacrylamide gel and subjected to either EDC based chemical crosslinking or traditional UV based crosslinking after electro-blotting to the nylon membrane

DISCUSSION

The variation of EDC-based chemical crosslinking was developed and used for first time by Giangrande et al. to detect weakly expressed microRNA from *Drosophilla melanogaster* whole embryos (Laneve and Giangrande, 2014). Here we, utilize the similar strategy to detect the population of small RNA species such as cytosolic methionine initiator tRNA and glycine tRNA isolated from HEK293T cells. It is clear from our results that EDC-based chemical crosslinking method exhibits increased sensitivity with an average of around 50-fold improvement of target detection.

One of the critical determinants of RNA-based technologies is the maintenance of the integrity of RNA. The ubiquitous presence of RNases limits the process of RNA manipulations because of enhanced chances of RNA degradation right after the extraction until gel loading. Therefore, all equipment, glassware/ plastic ware should be thoroughly wiped with RNase-based surface decontaminant and followed by rinsing with DEPC-treated water. All the solutions should be prepared by using DEPC-treated water as a solvent.

Residual DEPC or DEPC by-products have been known to interfere in several enzymatic reactions and is also known to modify RNA (carboxymethylation), therefore, DEPC should be inactivated and removed by autoclaving at 121°C for at least 30 to 40 min. Also, 10x TBE should not be prepared directly in active DEPC containing water. The presence of nucleophile amine groups of TRIS would hydrolyse and inactivate DEPC, therefore treatment against RNAse would not be complete. It is highly recommended to prepare 10x TBE in already autoclaved DEPC-treated water, followed by 2nd round of autoclaving.

The EDC mediated chemical crosslinking technology is based on the formation of covalent bond between RNA 5'monophosphate group and primary amines on nylon membrane. The involvement of only 5' head of RNA in crosslinking leaves the rest of RNA free for hybridization, which enhances the sensitivity of assay.

The sharpness of the band depends on several parameters. However, small sample volume (2-5 µL), thin gels, loading of sample from bottom of the well and removal of urea cushions from the well pockets ensure sharper and better resolved the RNA bands. It is also recommended to initially run at lower voltage (100 V for 30 min) or cast 4% stacking gel over separating gel, in order to enhance the sharpness of bands.

REFERENCES

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PRECAUTION

- Trizol contains phenol and guanidine thiocyanate, which are extremely corrosive to skin and should be handled with care and wearing protection gear such as gloves, lab coat and protection glasses. The place should be properly ventilated
- Acrylamide/bisacrylamide: Potent neurotoxin
- Ethyl bromide: Potent mutagen and carcinogen, DNA intercalating agent
- UV rays are toxic to skin and mutagen. Use proper shield to avoid exposure to UV
- 1-Methylimidazole: Corrosive to skin, causes serious eye damage, causes acute toxicity (oral, dermal, inhalation)
- HCl: Corrosive (dermal, inhalation). Concentrated solution releases toxic fumes
- Use gloves, face masks, lab coat and work under properly ventilated area
- ATP, [γ-32P]: Radioactive material which emits beta rays. Wear lab coat, hand gloves, safety spectacles while handling. Carefully, read and follow the recommended safety measures while handling radioactive material

CONFLICT OF INTEREST

The manuscript was reviewed even one of the authors is directly involved in the editorial team of this journal.

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