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TITLE:

RNA Blot Analysis for the Detection and Quantification of Plant MicroRNAs

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KEYWORDS:

Plant micro(mi)RNAs, RNA blot, denaturing PAGE, electroblotting, hybridization, quantification of miRNAs.

SUMMARY:

This method demonstrates use of the northern hybridization technique to detect miRNAs from total RNA extract.

ABSTRACT:

MicroRNAs (miRNAs) are a class of endogenously expressed non-coding, ~21 nt small RNAs involved in the regulation of gene expression in both plants and animals. Most miRNAs act as negative switches of gene expression targeting key genes. In plants, primary miRNAs (pri-miRNAs) transcripts are generated by RNA polymerase II, and they form varying lengths of stable stem-loop structures called pre-miRNAs. An endonuclease, Dicer-like1, processes the pre-miRNAs into miRNA-miRNA* duplexes. One of the strands from miRNA-miRNA* duplex is selected and loaded onto Argonaute 1 protein or its homologs to mediate the cleavage of target mRNAs. Although miRNAs are key signaling molecules, their detection is often carried out by less than optimal PCR-based methods instead of a sensitive northern blot analysis. We describe a simple, reliable, and extremely sensitive northern method that is ideal for the quantification of miRNA levels with very high sensitivity, literally from any plant tissue. Additionally, this method can be used to confirm the size, stability and the abundance of miRNAs and their precursors.

INTRODUCTION:

The recent discovery of small regulatory RNAs, microRNAs, has led research in understanding them and their role in plants and animals[1]. Long precursors of miRNAs are processed into 21 to 24 nt mature miRNAs by HYL1 and specific dicer-like proteins[2,3]. A 22 nt miRNA can initiate cascade silencing by generating secondary siRNAs[4]. Studies have shown the role of miRNAs and

secondary siRNAs in development, cell fate and stress responses[5,6].

Northern hybridization is an experimental method routinely employed to detect specific RNA molecules. This method customizes its use in the detection of approximately 19 -24 nt long small RNAs from a pool of total RNAs[7]. In this demonstration, we illustrate the use of this technique for the detection and quantification of miRNAs. This method uses labelling of probes using radioisotopes; thus, miRNA levels in the sample can be detected with increased sensitivity. Unlike PCR-based methods, this method ensures quantification of expression as well as size determination of the miRNAs. In this protocol, we show crucial steps that improve miRNA detection. We have modified steps in blotting and hybridization for obtaining high resolution signal detection of miRNAs. This technique can also be used for the detection other endogenous small RNAs such as siRNAs, tasi-secondary RNAs and snoRNAs.

PROTOCOL:

1. Preparation of a 15% denaturing polyacrylamide gel

1.1. Weigh and add 4.8 g of urea, add 3.75 mL of 40% acrylamide: bisacrylamide (19:1) solution and 1 mL of 10x TBE pH 8.2 into a sterile 50 mL tube.

1.2. Dissolve the urea using a water bath set at 60 °C into a clear solution.

1.3. Make-up the volume to 10 mL using freshly autoclaved sterile water and cool the gel mix to room temperature.

1.4. Prepare fresh 10% (w/v) ammonium persulfate solution.

2. Assembly of the glass plates and electrophoresis unit

2.1. Wash all the apparatus required for the gel electrophoresis and electro-blotting with detergent. Gently scrub them using a soft sponge to remove residual buffer and acrylamide, rinse with water and allow them to dry.

2.2. Assemble both the glass plates together and place them firmly on the sponge. Use a 1 mm thick plate for this setup. Make sure that the plates are at the same level to avoid leakage.

2.3. To the 10 mL gel mix, add 8 µL of TEMED and 80 µL of freshly prepared 10% (w/v) ammonium persulfate solution.

2.4. Without delay, gently mix and pour this in between the assembled glass plates. Place the comb carefully. Avoid generating air-bubbles in this step.

2.5. Allow the gel to polymerize for approximately 45 min.

2.6. Wash the polymerized gel with sterile water before placing inside the gel-running setup.

2.7. Assemble the plates inside the running cassette and remove the comb gently.

2.8. Pour freshly prepared sterile 1x TBE, pH 8.2 into the tank.

2.9. Wash the wells carefully by pipetting the buffer to remove salt crystals. This step helps the RNA sample to run uniformly across the gel.

2.10. Perform a pre-run at 80 V for 30 min.

3. Preparation of loading dye and samples

3.1. For 10 mL of gel loading dye, weigh 5 mg of bromophenol blue, 5 mg of xylene cyanol, add 10 mL of deionized formamide carefully and mix them well.

3.2. Aliquot 10 µg of total RNA into a sterile 1.5 mL tube and dry the samples using a speedvac. Do not over-dry the samples.

3.3. Resuspend the RNA samples in 8 µL of loading.

3.4. Heat the samples at 98 °C for 2 min, cool for 1 min at RT, vortex and spin the samples, for 3 times. This step is essential for proper resuspension and in turn this helps in equal loading of the samples.

4. Gel electrophoresis

4.1. Stop the pre-run and wash the wells before sample loading.

4.2. Heat the samples at 98 °C for 1 min and load the samples hot into the well using capillary tips. Insert the tip to the bottom of the well, so that sample occupies one thin layer in the well.

4.3. Complete loading of all the samples. Include to load RNA decade marker.

4.4. Run the gel at 80 V until the bromophenol blue dye runs almost completely. Bromophenol blue runs at 10 bp in a 15% denaturing acrylamide gel.

5. Preparation for electro-blotting

5.1. Cut the N+nylon membrane to the dimensions of glass plate and label the membrane at its top-right corner with a HB pencil.

5.2. Gently place the membrane on surface of sterile water, facing the labeled side towards water surface. Pre-soak the membrane for 15 min.

5.3. Cut 4 pieces of blotting paper I to the dimensions of fiber pad.

5.4. Prepare the gel sandwich for placing the gray side of the cassette down in a clean tray.

5.5. Pre-wet the fiber pad and place it over the cassette. Remove air bubbles.

5.6. Pre-wet one piece of blotting paper in 1x TBE and place over the fiber pad. Remove air bubbles by rolling a plastic pipette over the paper. Lay another piece of pre-wet blotting paper and remove air bubbles.

5.7. Carefully remove the gel from the running cassette and place it over the sandwich setup, such that the first loaded RNA sample is towards the right.

5.8. Gently dip the pre-soaked membrane in 1x TBE and place it over the gel, facing the labelled side down. Roll out to remove the air bubbles.

5.9. Dip a piece of blotting paper, lay it over the membrane, and remove the air bubbles. Dip another piece of blotting paper, place it over sandwich setup and remove air bubbles.

5.10. Complete the sandwich by placing a pre-wet fiber pad over the setup and firmly close the cassette.

5.11. Place the transblot cassette in the module and fill 1x TBE, pH 8.2 up to the blotting mark.

5.12. Transfer at 10 V, overnight at 4 °C.

5.13. After transfer, place the damp membrane on a paper sheet and immediately cross-link the RNA to the membrane by irradiation with 254-nm UV light (120,000 µjoules/cm^2). The cross-linked blot maybe stored at 4 °C for further hybridization.

6. Preparation of radiolabeled probe

6.1. Design a probe that is completely complementary to the small RNA which is to be detected.

6.2. End label the probe using YP^{32}ATP (obtained from BRIT) at its 5' end by combining the components as per recipe provided in **Table 1**.

6.3. Incubate the above reaction mixture at 37 °C for 30 min.

6.4. Separate un-labelled YP^{32}ATP from probe by using a Sephadex G-25 column according to manufacturer's protocol.

7. Hybridization of the blot

7.1. Place the crossed-linked blot, RNA side facing top inside a hybridization bottle.

7.2. Vigorously mix the ultra-sensitive hybridization buffer, add 10 mL of it and incubate the blot inside a hybridization oven maintained at 35 °C, with rotation.

7.3. Perform pre-hybridization for 20 min.

7.4. Remove the bottle from the oven and add the labelled probe into the hybridization buffer gently.

7.5. Hybridize the blot at 35 °C, with rotation for 12 h.

7.6. After hybridization carefully transfer the hybridization solution into 15 mL tube. This solution can be stored at 4 °C until re-use.

7.7. Perform a quick wash of the blot for 2 min to remove excess hybridization solution by adding 2x SSC buffer plus 0.5% SDS. Discard the solution.

7.8. Incubate the blot with 2x SSC buffer plus 0.5% SDS for 35 °C, with rotation for 30 min.

7.9. Wash the blot again with 0.5x SSC buffer plus 0.5% SDS for 35°C, with rotation for 30 min.

7.10. Place the blot inside a hybridization cover, remove the excess buffer and seal it.

7.11. Expose it to a radiation free-phosphor imager screen overnight inside a cassette.

7.12. Detect the hybridization signal using biomolecular imager and analyze the results using suitable software.

7.13. Strip the blot by incubating it at 80 °C, with rotation for 30 min with 0.5 X SSC buffer plus 0.1% SDS and 0.1 X SSC buffer plus 0.1% SDS for 30 min.

REPRESENTATIVE RESULTS:

In this demonstration, we have detected and quantified the expression of miR397 in different tissues of *indica* rice *var* whiteponni (**Figure 1**). miR397 is a 22 nt miRNA and conserved miRNA. The expression of miR397 can be detected in all the tested samples. As per the next-generation sequencing data, sample 1 (seedling tissue) has miR397 at 5 reads per million (rpm). We detected its signal comfortably, indicating that the method is very sensitive and can be used to detect even very low abundant miRNAs. In this experiment, we have used miR168 and *U6* as loading controls.

In this blot (**Figure 1**), strength of signals was quantified using ImageJ. The expression of miR397 is highest in vegetative leaf sheath.

FIGURE AND TABLE LEGENDS:

Figure 1: Detection and quantification of expression of miR397 in different tissues of rice samples.

Table 1: Table of recipes.

DISCUSSION:

This method can be extensively used for detection and quantification of small RNAs including less abundant miRNAs. The protocol mainly describes the steps for denaturing the total RNA in a loading buffer, size separation by gel electrophoresis, transfer of RNA to a membrane, cross-link the RNA onto membrane and hybridize using desired radiolabeled oligo probes.

The critical step for any blotting experiment is the use of good quality RNA for sample preparation. Before loading the gels, one must make sure that the samples are free flowing and not sticking to the loading tips. Care must be taken while loading the sample, tip should be inserted just above the bottom of the well so that sample occupies one thin layer in the well. The temperature of hybridization oven must be maintained at 35 °C for detection of miRNAs that are extremely less abundant. For repeated hybridization of blot, store the membrane at 4 °C by keeping it damp in 2x SSC.

This method can be used to detect small RNAs from tissues that are rich in polysaccharides and polyphenols[8]. In this protocol, usage of vacuum drying for concentrating RNA samples provides better stability and less loss of sample compared to other older methods[9]. Other modification in the method includes, spreading of the membrane in water before dipping in 1x TBE during electro-transfer. This improves the efficiency of RNA transfer, providing better resolution of blot.

A major limitation of the method is usage of radioisotopes, which needs trained personnel and a radioisotope lab to perform the hybridization experiments. This method here provides detailed information regarding all the steps involved in the RNA blot analysis for the detection of miRNAs. This protocol also ensures the size of the small RNA apart from its signal detection[10]. The technique provides a robust tool for molecular biologists to estimate the abundance of various small RNAs such as miRNAs, secondary siRNAs and snoRNAs.

ACKNOWLEDGMENTS:

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DISCLOSURES:

No conflicts of interests declared.

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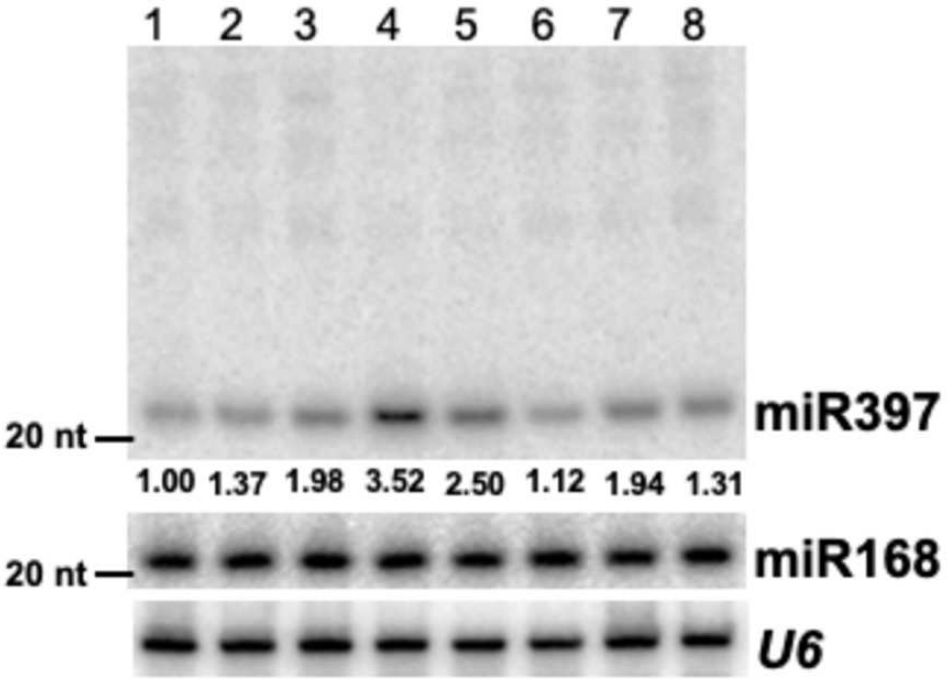
Detection and quantification of miR397 in different tissues of *indica* rice *var* Whiteponni



1: Seedling
2: Root
3: Stem
4: Vegetative leaf sheath
5: Vegetative leaf
6: Flag leaf
7: Reproductive leaf sheath
8: Flower

Strength of signals are quantified using ImageJ software

Table 1: Table of recipes

Buffer and solution	Recipe	Comments
10 X TBE	0.89M Tris buffer 0.89M Boric acid 30mM EDTA	pH should be set to 8.2 using acetic acid
Gel mix	15% acrylamide-bisacrylamide sol, 19:1 8M urea 1X TBE	Gel mix should not contain urea crystals
Gel loading dye	0.05 %(w/v) bromophenol blue 0.05 %(w/v) Xylene xyanol 100 % deionized- formamide	Care should be taken while handling deionized formamide
Labelling of probe	PNK buffer (10X, 2 µL) PNK enzyme (1 µL) Oligo (100 µM, 0.1 µL) γP^{32} ATP (4 µL)	This mixture contains radioactive molecules, this step must be performed by trained personel inside a radioactive lab
Wash Buffer - I	2X SSC 0.5% (w/v) SDS	
Wash Buffer - II	0.5X SSC 0.5% (w/v) SDS	
Stripping Buffer - I	0.5X SSC 0.1% (w/v) SDS	
Stripping Buffer - II	0.1X SSC 0.1% (w/v) SDS	

Table 2: Table of materials

Equipment	Company	Catalog no
40% Acrylamide-bisacrylamide solution	Sigma	A9926
Ammonium persulphate (APS)	BioRad	1610700
Blotting paper	whatmann blotting paper I	1001-125
Bromophenol blue	Sigma	B5525-5G
Capillary loading tips	BioRad	2239915
Deionized formamide	Ambion	AM9342
Heating block	Eppendorff	5350
Hybond N+nylon membrane	GE	RPN203B
Hybridization bottle	Sigma	Z374792-1EA
Hybridization Oven	Thermo Scientific	1211V79
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma	T7024-25ml
PhosphorImager	GE- Typhoon scanner	29187194
PhosphorImager screen and cassette	GE healthcare	GE28-9564-75
Pipettes	Gilson, models: P20 and P10	FA10002M, FA10003M
Plastic pipette	Falcon	357550
Polyacrylamide gel apparatus	BioRad	1658003EDU
Sephadex G-25 column	GE healthcare	27532501
Speed Vac Concentrator	Thermo Scientific	20-548-130
Spinwin	Tarsons	1010
T4 Polynucleotide Kinase (PNK)	NEB	M0201S
Transblot apparatus	BioRad	1703946
ULTRAHyb hybridization buffer	Ambion	AM8670
Urea	Fischer Scientific	15985
UV-crosslinker	UVP	CL-1000L
Vortex	Tarsons	3020
Water bath	NEOLAB	D-8810
Xylene cyanol	Sigma	X4126-10G

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. No changes are required.

Author's reply: Thank you. We have made some changes in the manuscript and have updated the tables, kindly incorporate these changes in the revised manuscript.

Changes to be made by the Author(s) regarding the video:

1. Please change the numbering of the protocol title slides to match the numbering in the written manuscript. Please use numbers instead of letters.

Author's reply: Necessary changes have been made in the title slides.

2. On-Screen Text and Title Cards:

• Please remove the phone number from the title cards.

"• 01:27 ""Step A: Preparation of a 15% denaturing polyacrylamide gel"" This chapter title card is a little glitchy and jerky. Please fix the animation so it is not distracting or simply remove the effect. Clarity over style.

• 02:02 ""Step B: Assembly of the glass pllates and electrophoresis unit"" This chapter title card is also a little glitchy/jerky. The style seen @04:13 (""Step C"") is a good example of a style that works."

"The symbol for ""micro"" as in ""microliters"" is Greek letter Mu lowercase: µ

This is not a lowercase U.

• 02:41 ""ul"" -> ""µL""

• 10:21 ""ul"" -> ""µL"""

•Instead of the Representative figure title card, please say Representative Results.

Author’s reply: The suggested changes have been made in the revised video.

3. Editing & Narration:

• 03:52 There is an audio pop after the words, "thoroughly by" You may be able to delete it.

• 12:31 There is a frame or two of the previous figure left when the imagining machine cuts to the stripping instructions.

Author’s reply: As per the suggestion, we have edited the video.