Isolation and detection of small RNA molecules

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ABSTRACT

The article describes in detail methodical procedures for isolation of small RNA molecules, their electrophoresis, northern blotting and finally detection by hybridization with radioactively labeled riboprobes. The presented protocols were successfully introduced and used in our laboratory.

Keywords: protocol; northern blotting; riboprobe

In many eukaryotes, including fission yeast, fruit fly, nematode *Caenorhabditis elegans*, mammals and plants, RNA interference pathway generates small RNA molecules which can direct epigenetic modifications that repress gene expression to complementary regions of the genome.

Up today there are several distinct classes of small RNA molecules. MicroRNAs (miRNAs) are ~21–22nt long molecules which are produced by Dicer family of endonuclease III enzymes from longer endogenous precursor RNAs that form stem-loop structures (Carrington and Ambros 2003, Bartel 2004). Small interfering RNAs (siRNAs) have two major size classes ~21 and ~24nt and are also generated by Dicer enzymes from long double-stranded RNAs (Baulcombe 2004, Matzke and Birchler 2005, Brodersen and Voinnet 2006). miRNAs and siRNAs are components of RNA-induced silencing complex (RISC) which is guided to complementary mRNAs causing translational inhibition or transcript cleavage (He and Hannon 2004, Meister and Tuschl 2004, Tang 2005). siRNAs of ~24nt length can also guide DNA and histone methylation leading to gene silencing (Baulcombe 2004, Bender 2004, Mello and Conte 2004, Chan et al. 2005, Matzke and Birchler 2005, Morris 2005). Piwi-interacting RNAs (piRNAs) are 25–31nt long molecules which were isolated from tests of mammals (Carthew 2006). Repeat associated small interfering RNAs (rasiRNAs) are 24–29nt long molecules isolated from *Drosophila* germline where they ensure genomic stability (Vagin et al. 2006). The mechanism of synthesis of piRNAs and rasiRNAs is not yet known.

Small RNAs are now subjects of intensive investigation. A common feature of all such RNAs is their size that fall within defined range between 19 and 31nt (Kim 2005, Zamore and Haley 2005, Carthew 2006). Small regulatory RNA species have discrete sizes and therefore are easily distinguishable from catabolic fragments of longer cellular RNAs.

Methodology for small RNA identification, validation and expression profiling is still improving (Berezikov et al. 2006). Widely used northern blot hybridization is less sensitive than RT-PCR-based techniques and has lower throughput than microarray expression analysis, cDNA cloning or sequencing. However, northern blot hybridization is inexpensive, technically easy to perform, quantitative and often used to validate small RNA identified by high throughput methods. siRNAs appear to lack precise precursors and piRNAs or rasiRNAs do not arise from transcription of inverted repeats; it is thus difficult to predict and validate these molecules by computer-based methods as for miRNAs (Sethupathy et al. 2006). In such cases, using candidate-sequence probes for hybridization of small RNA northern blots is a method of choice for discovery of new small

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RNA species. Northern blot hybridization can also be used to determine the amount and size of siRNA produced from transgenes introduced to knockdown genes and so can help validate these experiments.

The following chapters represent step-by-step procedures to isolate, northern blot and detect small RNA molecules, which were successfully introduced and used in our laboratory (Fulnecek and Kovarik 2007). The principles of small RNA cloning and the link to protocol are presented at the end of the article.

EXPERIMENTAL PROCEDURES

Extraction of small RNA molecules

The following protocol is based on Dr. Andrew J. Hamilton protocol "Extraction of 25NT RNA" from the David Baulcombe group, The Sainsbury laboratory (Hamilton and Baulcombe 1999) with modifications and improvements as cited in the text.

Avoid contamination by RNases using proper RNA handling. During whole procedure (isolation and detection), use RNase-free solutions and lab equipment and wear gloves.

Total RNA isolation. Homogenize fresh plant material (4 g of leaves, seedlings, etc.) in liquid nitrogen with a mortar and a pestle. Transfer homogenate into RLT buffer (2 ml/g, Qiagen) in 50-ml falcon tube (our modification). For homogenization it is also possible to use other guanidine thiocyanate buffers. Extract two times with phenol-chloroform (add one volume) and precipitate nucleic acids by adding 0.1 volume of 3M NaOAc pH 5.0 and 3 volumes of ethanol and incubating at –20°C for 2 hours. Recover the total RNA/DNA by centrifugation at 10 000 × g for 10 minutes. Wash the pellet by 75% ethanol and let it air-dry (Hamilton protocol).

For detection of abundant and known small RNA molecules RNA preparations from 100 mg of plant tissues using Trizol (Invitrogen) are sufficient (our observation).

Enrichment for small RNA. Dissolve the pellet in about 5 ml of TE (10mM Tris-Cl pH 7.5, 1mM EDTA; for Trizol preparations only ~0.1 ml), heat this to 65°C (faster dissolving of the pellet and disrupting any association of small RNA with DNA). Place the solution on ice and add PEG (M_w = 8 000) to a final concentration of 5% and NaCl to a final concentration of 0.5M. Mix well and incubate on ice for 30 minutes. Spin down high molecular weight nucleic acids at 10 000 × g for 10 minutes. Transfer supernatant to a new falcon tube and precipitate small RNA molecules by adding 3 volumes of ethanol. Place the mixture at –20°C for at least 2 hours and then spin down the RNA at 10 000 × g for 10 minutes. Wash the pellet by 75% ethanol and let it air-dry. Dissolve this preparation in formamide (0.1–0.5 ml), quantify RNA by measuring UV absorbance of aliquot and use 5–50 μg of small RNA directly for northern blots (Hamilton protocol). However, aberrant migration during electrophoresis can be noticed sometimes, and that is why further purification of small RNA fraction may be necessary.

Purification of small RNA fraction. Equilibrate DEAE-Sepharose CL-6b (Sigma) in buffer A (50mM MOPS/NaOH pH 7, 15% isopropanol, 0.2M NaCl, 0.15% Triton X100). Prepare column by plugging the end of a 5 ml disposable syringe by WHATMAN No. 1 filter paper circles and adding 1 ml of the equilibrated sepharose. Let the column settle, dissolve the pellet of small RNA in buffer. A and apply to the column under force of gravity. Collect the flow-through and apply to the column again. Wash the column with 10 volumes of buffer A and elute small RNA with buffer B (similar as buffer A, only concentration of NaCl is 1M). Add 3 volumes of ethanol to the eluate, mix, place it at –20°C for 2 hours and spin down the small RNA at 10 000 × g for 10 minutes. After washing the pellet with 75% ethanol and air-drying dissolve it in formamide (0.1–0.5 ml) (Hamilton protocol).

Electrophoresis and northern blotting

Separate small RNA molecules in 15% polyacrylamide (19:1), 50% urea, 1 × TBE (90mM Tris-borate, 2mM EDTA) gels. Apparatus with 20 cm long and 1.5 mm thick gel is sufficient (our observation). It is usually possible to separate up to 50 μg of small RNA in one lane. Prepare one additional sample to which add one or two radioactively end-labeled DNA oligonucleotides (about 20 and 25 bases) (our modification) or radioactively end-labeled RNA ladder (10–150 bases, Ambion Decade Markers) to serve as length standards. As a positive control, load additional samples supplemented with a range of concentration (1 fmol–1 pmol) of ~20nt DNA oligonucleotide complementary to probe (our modification). As a negative control, use small RNA samples prepared from different plant species (our modification). Add bromophenol blue

and TBE buffer to samples dissolved in formamide (recommended final concentration of formamide is at least 50%), heat it at 65°C for 5 minutes, place on ice and load the gel. Run the gel until bromophenol blue reaches the bottom of the gel (our observation). Wash the gel in 0.5 × TBE (10 min), stain RNA with ethidiumbromide in 0.5 × TBE (10 min) and then wash the gel again in 0.5 × TBE (10 min). Photograph the gel in order to document the RNA quantity in individual lanes and cut off the part of the gel with overloaded tRNAs and 5S rRNA molecules (from start to about 80nt). Blot the RNA to nylon membrane (Hybond N+, TM or other positively charged membrane useful for northern blotting of small RNA molecules) at 3 mA/cm^2 for 1 hour in 0.5 × TBE using semi-dry blot apparatus. Carefully take the membrane, do not wash it, fix the RNA either by placing the membrane on filter paper soaked in 0.5 × TBE and UV crosslinking or by baking the membrane at 80°C for 2 hours (our modification). Store the dry membrane in dark until use.

Detection with radioactively labeled probe

Detection of known and abundant small RNA molecules (several pg per lane) is possible by hybridization with complementary 5' end labeled oligonucleotide. T4 Polynucleotide Kinase (~30 units) and [γ-^{32}P] ATP (2–5 fold molar excess to 1–10 pmol of an oligonucleotide) are used to label an oligonucleotide to high specific activity (Sambrook and Russell 2001). Radiolabeled oligonucleotides can be purified either by precipitation with ethanol (Sambrook and Russell 2001) or by gel filtration on columns (NucAway-Spin Columns, Ambion).

Preparing the riboprobe. If you do not know the exact sequence of small RNA of interest (small RNA molecules were not cloned and sequenced) or there is limited amount of target small RNA in sample for detection by northern blot hybridization (~10 fmol per lane) you have to prepare strand-specific single-stranded RNA probe (riboprobe) by incorporation of [α-^{32}P] UTP or GTP in *in vitro* transcription reaction with bacteriophage RNA polymerase (Sambrook and Russell 2001). Riboprobe is prepared from linearised recombinant plasmid that carries bacteriophage promoter immediately upstream of the DNA fragment of interest or by adding the promoter to 5' end of DNA fragment using PCR (Sambrook and Russell 2001). Check on the orientation of promoter and the sense of generating RNA strand. Use DNA template with blunt or 5'-protruding ends because 3'-protruding termini can aberrantly initiate transcription (blunt the ends of PCR product of Taq DNA polymerase). Prepare RNase-free DNA template (treatment with proteinase K and phenol-chloroform extraction is recommended).

Assemble *in vitro* transcription reaction in laboratory according to the protocol (Sambrook and Russell 2001) or use commercial kit (RNAMaxx High Yield Transcription Kit, Stratagene). Use up to 10 µl of [α-^{32}P] rUTP (10 mCi/ml, 3 000 Ci/mmole), transcription buffer, 0.5mM each rATP, rGTP, rCTP, 0.2–0.5 µg of template DNA, 30mM dithiothreitol, 0.3U of yeast inorganic pyrophosphatase, 1 µl of RNase block and 200U of RNA polymerase per 20 µl reaction volume (our modification). Incubate the complete reaction mixture at 37°C for 2 hours. Terminate the reaction and digest the template DNA by incubation of the reaction mixture with 1 µl of RNase-free DNaseI at 37°C for 30 minutes. Two times precipitate the riboprobe with ethanol – add 10 µl of 7.5M NH_4Ac, mix, add 75 µl of ethanol, mix, keep on ice for 10 minutes, spin at 15 000 × g for 15 minutes, resuspend a pellet in 100 µl TE, add 50 µl of 7.5M NH_4Ac, mix, add 375 µl of ethanol, mix, keep on ice for 10 minutes, spin at 15 000 × g for 15 minutes, wash the RNA pellet with 75% ethanol, let it air-dry for 5 minutes and resuspend it in 20 µl of TE (according to the Stratagene RNAMaxx High Yield Transcription Kit protocol).

Hybridization of nucleic acids fragments of comparable length can avoid high background and has a higher stability of RNA-RNA homoduplexes. Therefore, prior to hybridization, long riboprobes are statistically fragmented to 50nt long molecules to obtain pool of fragments with different sequences. Add 300 µl of 200mM Carbonate solution (80mM $NaHCO_3$, 120mM Na_2CO_3) per 20 µl of transcription reaction and incubate it at 60°C for *t* minutes, which is the time necessary for riboprobe fragmentation to an average size of ~50 nucleotides:

$$t = (L_i - L_f)/(K \times L_i \times L_f)$$

where: t is the time in minutes, L_i is the initial length of the probe in kb, L_f is the final length of the probe in kb (0.05 for 50nt) and K is the rate constant (K = 0.11 kb/min)

At the end of the incubation add 20 µl of 3M NaOAc (pH 5) to stop the reaction (Hamilton protocol). It is possible to add the mixture directly

to the hybridization solution (5–10 ml of hybridization solution per small hybridization bottle).

Prepare "cold" riboprobe and analyze it on agarose gel to check on the efficient transcription of the template DNA before using [α-^{32}P] UTP. During preparation of radioactive riboprobe take aliquots of the reaction mixture for analysis in case of problems with detection of small RNA to monitor potential mistakes during probe preparation (our modification).

Hybridization. Prehybridize the membrane in hybridization buffer for northern blots [including 50% deionized formamide and 7% SDS, (Sambrook and Russell 2001)] at 40°C for at least 1 hour. It is possible to use UltraHyb-Oligo (Ambion) (our observation). After adding the probe, hybridize the membrane 1–3 days at 30°C. Wash the membrane two times with 2 × SSC, 0.5% SDS at 30°C for 10 minutes. Wrap the wet membrane in a saran wrap and expose it in a phosphorimage cassette for 1 hour or overnight (depends on radioactivity of the membrane, our modification). It is possible to decrease background (in case of riboprobe) by stripping the membrane with RNaseA at room temperature for 10 minutes (the stripping solution is 20mM Tris-Cl pH 7.5, 5mM EDTA, 60mM NaCl, 2 μg/ml RNase A) (Mette et al. 2000). This step can be repeated until the background signal is low.

Cloning of small RNA molecules

Another way to detect small RNA molecules is extraction of all small RNA molecules from denaturing polyacrylamide gel ("Crush and soak"; Sambrook and Russell 2001), cloning and sequencing. There are many protocols, for example: http://www.tsl.ac.uk/dcb/services/Small_RNA_cloning_protocol.pdf from The David Baulcombe group, The Sainsbury laboratory (Chappell et al. 2005). The principle of the protocol is as follows: Hybrid 5'-DNA-RNA-3' adaptor is ligated to 5' ends of small RNA molecules using T4 RNA ligase. Phosphorylated hybrid 5'-RNA-DNA-3' adaptor with blocked 3'-hydroxyl terminus is ligated to 3' ends of small RNA molecules. Final ligation products are reverse transcribed and amplified. The PCR product is digested with restriction enzymes in adaptor regions and concatemerised using T4 DNA ligase. The concatemers are ligated into cloning vector, recombinant plasmids are cloned, inserts are amplified and sequenced and from the data small RNA sequences are extracted manually or automatically using software tools.

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REFERENCES

Bartel D.P. (2004): MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, *116*: 281–297.

Baulcombe D. (2004): RNA silencing in plants. Nature, *431*: 356–363.

Bender J. (2004): Chromatin-based silencing mechanisms. Curr. Opin. Plant Biol., *7*: 521–526.

Berezikov E., Cuppen E., Plasterk R.H. (2006): Approaches to microRNA discovery. Nat. Genet., *38*: S2–7.

Brodersen P., Voinnet O. (2006): The diversity of RNA silencing pathways in plants. Trends Genet., *22*: 268–280.

Carrington J.C., Ambros V. (2003): Role of microRNAs in plant and animal development. Science, *301*: 336–338.

Carthew R.W. (2006): Molecular biology. A new RNA dimension to genome control. Science, *313*: 305–306.

Chan S.W., Henderson I.R., Jacobsen S.E. (2005): Gardening the genome: DNA methylation in *Arabidopsis thaliana*. Nat. Rev. Genet., *6*: 351–360.

Chappell L., Baulcombe D., Molnár A. (2005): Isolation and cloning of small RNAs from virus-infected plants. In: Coico R., Kowalik T., Quarles J.M., Stevenson B., Taylor R.K., Simon A.E., Downey T. (eds.): Current Protocols in Microbiology. John Wiley & Sons, Hoboken, NJ.

Fulnecek J., Kovarik A. (2007): Low abundant spacer 5S rRNA transcripts are frequently polyadenylated in *Nicotiana*. Mol. Genet. Genomics. (In print)

Hamilton A.J., Baulcombe D.C. (1999): A species of small antisense RNA in posttranscriptional gene silencing in plants. Science, *286*: 950–952.

He L., Hannon G.J. (2004): MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet., *5*: 522–531.

Kim V.N. (2005): Small RNAs: classification, biogenesis, and function. Mol. Cells, *19*: 1–15.

Matzke M.A., Birchler J.A. (2005): RNAi-mediated pathways in the nucleus. Nat. Rev. Genet., *6*: 24–35.

Meister G., Tuschl T. (2004): Mechanisms of gene silencing by double-stranded RNA. Nature, *431*: 343–349.

Mello C.C., Conte D. Jr. (2004): Revealing the world of RNA interference. Nature, *431*: 338–342.

Mette M.F., Aufsatz W., van der Winden J., Matzke M.A., Matzke A.J.M. (2000): Transcriptional silenc-

ing and promoter methylation triggered by double-stranded RNA. EMBO J., *19*: 5194–5201.

Morris K.V. (2005): siRNA-mediated transcriptional gene silencing: the potential mechanism and a possible role in the histone code. Cell. Mol. Life Sci., *62*: 3057–3066.

Sambrook J., Russell D.W. (2001): Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sethupathy P., Megraw M., Hatzigeorgiou A.G. (2006): A guide through present computational approaches for the identification of mammalian microRNA targets. Nat. Methods, *3*: 881–886.

Tang G. (2005): siRNA and miRNA: an insight into RISCs. Trends Biochem. Sci., *30*: 106–114.

Vagin V.V., Sigova A., Li C., Seitz H., Gvozdev V., Zamore P.D. (2006): A distinct small RNA pathway silences selfish genetic elements in the germline. Science, *313*: 320–324.

Zamore P.D., Haley B. (2005): Ribo-gnome: the big world of small RNAs. Science, *309*: 1519–1524.

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