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Analysis of post-transcriptional RNA metabolism in prokaryotes

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Abstract

Post-transcriptional RNA metabolic pathways play important roles in permitting prokaryotes to operate under a variety of environmental conditions. Although significant progress has been made during the last decade in deciphering RNA processing pathways in a number of bacteria, a complete understanding of post-transcriptional RNA metabolism in any single microorganism is far from reality. Here we describe multiple experimental approaches that can be used to study mRNA stability, tRNA and rRNA processing, sRNA metabolism, and polyadenylation in prokaryotes. The methods described here can be readily utilized in both Gram-negative and Gram-positive bacteria with simple modifications.

Keywords

RNA isolation; mRNA decay; tRNA processing; northern analysis; cleavage specificity; SMART cDNA; RNAseq

1. Introduction

RNA is a versatile biological molecule that can participate in a variety of critical functions in all living systems. However, unlike DNA, it is less stable and some RNAs, particularly mRNAs, turnover rapidly in prokaryotes. In *Escherichia coli*, the half-lives of mRNAs can vary from less than a minute to over 10 minutes [1, 2], whereas in *B. subtilis* half-lives of over 15 minutes are observed [3]. In contrast, the half-lives of non-coding rRNAs and tRNAs are over three hours in length unless they are defective, in which case they are rapidly degraded. The dynamic nature of RNA turnover ensures that microorganisms can quickly adapt to sudden environmental changes by modulating the rate of synthesis of either existing or new proteins. In fact, these adaptations can be attractive targets for developing novel antimicrobials, since the basis of host-pathogen interactions very often depends on changes in environmental conditions.

The majority of the basic techniques used to study various aspects of RNA metabolism have not changed significantly over last two decades, although a number of modifications have

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made them more robust and reliable. One area where there has been a significant improvement relates to the isolation of high quality RNA at relatively low cost. For example, the development of a simple, rapid, highly quantitative and inexpensive RNA isolation method [4] has made working with RNA more broadly accessible.

The Northern blot technique has not changed significantly and is still widely used for *in vivo* characterization of RNAs, but imaging techniques have significantly improved. In addition, while primer extension analysis was once the best and probably the only choice for mapping the precise 5' ends of either primary transcripts or processing and degradation intermediates, 5' end mapping can now also be accomplished by RT-PCR cloning and sequencing of 5' → 3' ligated transcripts [5] or by the cloning and sequencing of SMART™ cDNAs [6].

The addition of rifampicin to growing cultures to block new transcription initiation by RNA polymerase is still the method of choice for measuring the chemical half-life of specific mRNAs. While rifampicin is highly effective in inhibiting transcription from most of the mRNAs in *E. coli* with only a few exceptions [2], many tRNA levels keep increasing after rifampicin addition (Mohanty & Kushner, unpublished results). Similarly, it is possible that rifampicin may not be effective in arresting the transcription of all genes in other microorganisms.

A major advance in the analysis of RNA metabolism at the genomewide level has been the use of either high density tiling arrays [7] or the RNAseq procedure [8]. In recent years, the RNAseq procedure has undergone significant modifications to address specific questions, which include, but are not limited to profiling of gene expression, identification of novel transcripts, identification of transcription start sites (TSS), annotation of genes and/or operons etc. [9, 10]. Although still relatively expensive, the RNAseq method, along with various modifications designed to provide specific information about 5' and 3' termini [11-13], provides the most definitive view of transcriptome-wide RNA metabolism.

It should be kept in mind that each of the techniques discussed has its own advantages and disadvantages. Based on our experience analyzing various aspects of post-transcriptional RNA metabolism in prokaryotes over the past 30 years, we describe here the various procedures we routinely use in our laboratory, which have either been developed by us or have been adapted from techniques developed by other researchers in the field. We also discuss the strengths and weaknesses of each method.

2. Setting up for RNA analysis

2.1. Maintaining an RNase free environment

Because of its inherent lability, working with RNA requires meticulous handling of any item the RNA samples will come in contact with. Any laboratory that carries out plasmid DNA isolations has probably used RNase A to remove RNA from their preparations. Since RNase A is a remarkably robust enzyme that binds tightly to glassware and cannot be completely inactivated by steam sterilization, its use in the laboratory presents a major problem in terms of working with RNA. Therefore, the first step in setting up an RNase free environment is to segregate all items that will be used for RNA related experiments.

All glassware used for RNA should be sterilized by baking at 180°C for 6-8 hrs prior to use. All microfuge tubes and pipette tips should be purchased RNase free and used without steam sterilization. All other equipment (i.e. plastic ware, gel apparatus, etc.) should be made RNase free by treating with RNaseZap® (Ambion®) solution followed by thorough washing with regular deionized water and subsequently with RNase free water. We use the Synergy UV water purification system (Millipore) as source of RNase free water. Wearing powder free gloves at all times while working with RNA samples is strongly recommended.

3. RNA isolation procedures

The very first step in understanding any RNA metabolic pathway requires the quantitative isolation of good quality RNA that accurately reflects the *in vivo* collection of transcripts. In fact, lack of reliable and suitable RNA extraction procedures years ago was considered a major bottleneck for RNA related studies. Recently, several companies have developed a variety of RNA isolation kits, which can deliver good quality RNAs with a reasonable amount of effort. However, the cost of such kits may be prohibitive for many smaller laboratories, particularly in countries where there is limited support for research. Most importantly, many of the kits have limitations involving the size of the transcripts that can be isolated. For example, the size ranges of transcripts that can be isolated using the kits from some of the leading Biotech companies (Qiagen, Ambion®, Invitrogen etc.) are from 200 to >5700 nucleotides [4]. Considering that a significant fraction of the RNA population is below 200 nucleotides, which includes tRNAs and small regulatory RNAs (sRNA), a second kit specific for small molecular weight RNAs (such as Trizol® Max™ from Invitrogen) has to be used. In contrast, by using the RNA *snap*™ RNA isolation method [4] a broad size range of RNAs (<76 to >5700 nucleotides) are quantitatively isolated in a reproducible manner at a fraction of the cost compared to commercially available kits. While the RNA isolation procedure described below is primarily used in our laboratory for *E. coli*, the method has been used successfully in a variety of microorganisms by other researchers [14-19].

3.1. Protocol

The procedure described below has been modified from the previously published method [4] and works equally well on cells isolated from either exponential or stationary phase cultures.

3.1.1. Bacterial growth—The *E. coli* strains are routinely grown in either Luria broth or a suitable minimal medium by adding 2-5% of inoculum from an overnight grown culture. Bacterial cells are grown in a water bath shaker at an appropriate temperature. For strains containing temperature sensitive mutations the initial growth is carried out at 30°C until the cultures enter early exponential phase, whereupon they are shifted to 44°C for between 30 min –2 hrs depending on the particular mutation being studied. Cells are diluted periodically with appropriately prewarmed medium to maintain exponential growth. Cells from exponentially growing (5 ml) or stationary phase (0.7 ml) cultures are used for RNA isolation.

3.1.2. Steady-state RNA isolation—Steady-state RNA is used to compare the level of specific transcripts among different strains. All samples (5 ml each) are removed at early to mid-exponential growth and processed immediately as described below (section 3.1.4).

3.1.3. RNA half-life determination—Determination of chemical half-lives of RNAs for either genome-wide or specific transcripts requires inhibition of transcription initiation by adding rifampicin followed by isolation of total RNA at various time points. Rifampicin (500 µg/ml, dissolved in DMSO) and nalidixic acid (40 µg/ml, dissolved in water) are added to the cultures growing in the mid-exponential phase. At least 60-75 seconds are allowed for complete inhibition of transcription initiation to occur before the first sample (0 minute) is removed. Typically, an additional four to five samples are collected at regular time intervals within 20-30 minutes after rifampicin addition. Additional time points will not increase the accuracy of the half-life measurements. However, at least three biological replicates are recommended to increase the confidence in the half-life determinations. All samples are immediately added to an equal volume of stop buffer (SB) (Appendix 1) as described previously [20]. The SB prevents *in vivo* metabolic activity including RNase activity while waiting for all samples to be collected.

3.1.4. RNA extraction—The bacterial samples are centrifuged at 5000 rpm for 5 minutes and the spent medium is completely removed. Each cell pellet is completely resuspended thoroughly in 100 µl of RNA extraction solution (Appendix 1) and transferred to a microfuge tube. The cell suspensions are then heated at 95°C in a sand bath for 7 minutes. Carrying out this step in a water bath will not yield good results. Each sample is vortexed briefly and centrifuged in a microfuge at 16000 × g for 5 minutes. Ninety µl of the supernatant is carefully removed to a new microfuge tube without disturbing the loose pellet of cell debris.

3.1.5. Purification of RNA samples—The RNA isolated in previous step (section 3.1.4) is in formamide and can be used directly for Northern analysis. However, many RNA manipulation techniques require RNA to be resuspended in water. Thus, the RNA samples are further purified using a phenol/chloroform extraction (section 4.1), followed by sodium acetate and ethanol precipitation (section 4.2). The RNA sample (90 µl) is diluted thoroughly by mixing with 340 µl of RNase free water and extracted once with 430 µl of acid phenol/chloroform (section 4.1). The RNA in the aqueous phase is precipitated using the sodium acetate/ethanol procedure (section 4.2). The dried RNA pellet is then resuspended in 90 µl of RNase free water.

3.1.6. DNase I treatment—The RNA obtained from section 3.1.5 should be further treated with DNase I to eliminate any DNA contamination. This step is critical if the RNA is to be used for any experiment involving reverse transcription, such as RT-PCR and RNAseq experiments. Ten µl of 10X DNase I buffer (supplied by NEB) is added to 90 µl of RNA followed by 10 units (1 µl) of DNase I (RNase free, NEB) and incubated at 37°C for 30 minutes. The DNase I is inactivated and removed by extracting the reaction mixture once with phenol/chloroform (section 4.1). A second phenol-chloroform extraction is

recommended for RNAseq. The RNA is precipitated using sodium acetate/ethanol (section 4.2) and the RNA pellet is resuspended in 75-90 μ l of RNase free water.

3.2. RNA quantification

The RNA is initially quantified by measuring its optical density at 260 nm. We use a Nanodrop (Model 2000C, Thermo Scientific) for quantification and initial quality assessment of RNA samples. Comparable spectrophotometers can also be used. A good quality RNA should meet the criteria of a UV absorption ratio at 260/280 of >1.9 and at 260/230 of >2. We routinely obtain 800-1000 ng/ μ l of RNA from $\sim 5 \times 10^8$ cells using the above described method.

3.3. Gel analysis for quality assessment

After initial quality assessment by Nanodrop analysis, the physical integrity of the RNA samples is verified by gel electrophoresis using a mini agarose gel [20]. RNA samples (200-300 ng based on Nanodrop quantification) are separated on 1% agarose minigel in TAE (Tris-Acetate-EDTA) buffer (Appendix 1). Representative RNA samples isolated using above method are shown in Fig. 1.

4. Common procedures for purification and concentration of RNA

4.1. Phenol/chloroform extraction

Phenol/chloroform extraction is routinely used for purifying nucleic acids away from associated proteins and other contaminants. The RNA sample is thoroughly mixed by vortexing with equal volume of acid phenol/chloroform (5:1, pH 4.5, Ambion®). The resulting emulsion is centrifuged at full speed in a microfuge for 3 minutes to separate the aqueous layer from the organic phase. The aqueous phase (usually less than the starting volume) is transferred to a fresh microfuge tube. Care should be taken not to disturb the interphase which usually contains the protein contaminants.

4.2. RNA precipitation

Nucleic acids are routinely concentrated into a desired volume of water by using sodium acetate/ethanol precipitation. RNA in solution (such as the aqueous phase after phenol chloroform extraction as in section 4.1) is precipitated by first adding 0.11 volume of sodium acetate (3M, pH 5.2), followed by 2.5 volume of 100% ethanol. The mixture is incubated at either -80°C for one hour or -20°C overnight, which facilitates complete RNA precipitation. The solution is then centrifuged at full speed in a microfuge for 15 minutes in a cold room to form an RNA pellet. The supernatant is removed either by pipetting or aspiration. The RNA pellet is resuspended in 200 μ l of 70% ethanol followed by centrifugation at full speed in the microfuge for 5 minutes in the cold room. The ethanol is removed either by aspiration or pipette and the RNA pellet is air dried. It is important that the RNA pellet should not be over dried, since this will make the pellet harder to dissolve. The dried RNA pellet is resuspended in the desired volume of RNase free water.

5. Northern blot analysis

Northern blot analysis is the only currently available technique to analyze full-length RNAs without manipulation, both quantitatively and qualitatively. However, a major weakness of this method is that it may not be sensitive enough to detect transcripts with either very short half-lives (< 30 sec) or very low abundance. In these cases, real-time RT-PCR can be a good alternative. However, since the real-time RT-PCR method relies on amplifying a very short segment of a particular transcript, it will not provide an accurate assessment regarding the fate or integrity of the full-length transcript. Nevertheless, this method can successfully be used for smaller transcripts by employing appropriate statistical analysis [21]. In addition, half-life determinations employing real-time RT PCR can both be expensive and time consuming.

A detailed step by step procedure for Northern blot analysis has been described previously [22] and is presented in Appendix 2.

6. Analysis of 5' and 3' ends of RNA species

Analysis of the 5' and 3' termini of an individual RNA molecule provides valuable information regarding the origin and the nature of its post-transcriptional processing. It also helps in determining any post-transcriptional modifications that have occurred at either end of the transcript. We routinely use the following methods to identify either the 5' or both 5' and 3' ends simultaneously of various transcripts.

6.1. Analysis of 5' end of transcripts by primer extension

This is one of the earliest methods used to map the 5' end of a primary transcript or cleavage site of a processed transcript. We have regularly used this method to identify the 5' ends of variety of transcripts [1, 20, 23, 24].

6.1.1. Protocol—This procedure requires the use of radioisotope in order to achieve high quality results. Thus, appropriate safety precautions and rules must be followed when carrying out this procedure. Particular attention should be paid to not generating aerosols.

6.1.1.1. End-labelling of primer: A suitable primer complementary to the transcript of interest is designed to hybridize at ~ 50-100 nucleotides downstream of the expected 5' end. In our experience, a primer between 18-25 nt length (a longer primer does not provide any significant advantage) with at least 50% or higher GC content works best for primer extensions, but a primer with its first nucleotide as a G usually labels poorly with γ -[^{32}P]-ATP and thus should be avoided. The end-labelling reaction is set up as described in Appendix 3.

6.1.1.2. Reverse transcription: The reverse transcription reaction is carried out in a 20 μl reaction volume using SuperscriptTM III reverse transcriptase (Thermo Fisher). Initially, two μl of the end-labelled primer (section 6.1.1.1) is added to 1-20 μg of total RNA in a 12.5 μl reaction volume and heated in a 65°C water bath for 5 minutes to denature the RNA. The reaction is quickly chilled on ice for one minute and centrifuged briefly to collect all the

liquid at the bottom of the tube. All samples are held on ice until the following components are added in order: 5X First Strand buffer (provided by Thermo Fisher), 4 µl; 0.1M DTT, 1 µl; 10 mM dNTPs (dATP, dTTP, dGTP and dCTP), 1 µl; Optizyme Recombinant ribonuclease inhibitor (Fisher Scientific), 0.5 µl (20 units) and Superscript™ III, 1 µl (200 units). The primer extension is then carried out at 50°C for 1 hour. The reaction is then terminated by heating at 70°C for 15 minutes. The samples are quickly chilled on ice for one minute and centrifuged briefly to collect all the liquids at the bottom of the tubes followed by addition of 80 µl of sterile water to each tube to increase the reaction volume to 100 µl to minimize the loss of cDNAs during phenol/chloroform extraction.

The cDNAs are purified by extracting with 100 µl of phenol/chloroform solution (section 4.1). Ninety µl of the aqueous phase solution is transferred to a new microfuge tube. The cDNAs are concentrated by sodium acetate/ethanol precipitation (section 4.2) with following additional step. Before adding 2.5 volume of ethanol, one µl GlycoBlue™ (Ambion) is added to the solution which enhances the detection as well as the recovery of small amounts of cDNAs. The blue cDNA pellet is resuspended in 2.5 µl of Stop Solution from the Silver sequence™ DNA sequencing kit (Promega).

6.1.1.3. Running a sequencing gel and analysis: A DNA sequencing ladder is run along with the cDNA to map the 5' end of the cDNA. Initially, a template for the sequencing ladder is prepared by amplifying the genomic DNA containing the gene coding for the transcript of interest. The sequencing ladder is generated from the PCR DNA template by employing the same end-labelled primer used for reverse transcription (section 6.1.1.2) using the Silver sequence™ DNA sequencing kit (Promega) according to the manufacturer instructions. Both the sequencing ladder and the cDNA are separated on a 6% PAGE containing 8M urea at 50°C. The gel is run until 5-10 minutes after the loading dye runs off, which will remove most of the labelled primers. If the RT primer is more than 100 nt downstream of the expected 5' end, a second loading of the reactions may be required.

The gel is dried over a piece of Whatman blotting paper using a gel drier (Savant) for subsequent analysis. The gel is either scanned using a PhosphorImager or exposed to an X-ray film to obtain an autoradiograph. The position of each cDNA band representing a 5' end (either primary or processed) is determined based on its alignment to the corresponding nucleotide (G, A, T, C) band in the sequencing ladder. A representative primer extension is shown in Figure 2.

6.2. Analysis of 5' ends by SMART™cDNA

The primer extension method for the analysis of 5' termini of transcripts is a rapid and very useful method when target transcripts are unique without any sequence overlap with other similar transcripts. However, this method may not be suitable for transcripts with significant similarities in nucleotide sequences. For example, *E. coli* rRNAs (16S, 23S and 5S) are distributed among seven separate ribosomal operons. While the mature rRNA sequences are identical, the upstream and downstream sequences are highly heterogeneous amongst the seven operons making it almost impossible to differentiate the origin of each of the rRNAs in a primer extension analysis. Similarly, many tRNAs have several isotypes that differ

significantly in their sequences. Thus, a primer extension analysis, which cannot separate transcript isotypes based on their differences in nucleotide sequences, will lead to ambiguous results. In contrast, the SMART™ (switching mechanism at the 5' end of the RNA transcript) [6] approach is a very useful method under these circumstances, since it can distinguish individual transcript isotypes based on upstream sequences. However, this method is time consuming compared to primer extension analysis.

6.2.1. Protocol—The SMART™ technology takes advantage of the terminal transferase (TdT) activity associated with specific reverse transcriptases that usually add untemplated additional nucleotides (mostly dC residues) }, once the enzyme reaches the 5' end of the template strand it is copying. Such a property enables a template switching primer (the primers are described in detail in section 6.2.1.1) containing three consecutive rG nucleotides at its 3' end to complement the three dC nucleotides at the 5' end of the cDNA and serve to extend the initial template such that the reverse transcription will continue past the 5' end of the initial template (Fig. 3). Once the RNA template is degraded using RNase H, the single-stranded cDNA strand is converted into a double-stranded cDNA using the gene specific primer and an anchor primer. Subsequently, the double-stranded cDNA is cloned using restriction sites introduced through the two primers into a suitable vector (Fig. 3). The cloned cDNA is sequenced to determine an accurate representation of the original 5' end of the RNA transcript.

6.2.1.1. Primer design: P1 is the gene specific RT primer. This primer is designed ~50-100 nt downstream of the putative 5' end. The primer is 25-30 nt long (longer primers have no particular advantage) where the last 20 nucleotides at the 3' end are complementary to the transcript to be mapped. The complementary sequences should have a G/C content of at least 50% or more. A suitable restriction site is introduced into the first 10 nucleotides at the 5' end of the primer. P2 [5'CGCTGCGGCCGCGAATTCCGACCTCGAGC(*GGG*)3'] is the SMART template switching primer. The underlined sequences contain multiple restriction sites and the italicized three Gs are ribonucleotides. P3 (CGCTGCGGCCGCGAATTCCGACCTCGAGC) is the anchor primer.

6.2.1.2. cDNA synthesis using template switching: The overall procedure is as described in section 6.1.1.2 with the following modifications. The important distinction is that this method requires the use of a reverse transcriptase with TdT activity. We routinely use Superscript™ II for this procedure since it has TdT activity. Both primers P1 and P2 are added to the total RNA (1-5 µg) during the initial heat denaturation at 65°C. cDNA synthesis is carried out at 42°C for 60 minutes by adding 1 µl (200 units) of Superscript II™. After termination of cDNA synthesis by inactivating the Superscript II™ reverse transcriptase at 70°C for 15 minutes, the RNA template is degraded by treating the reaction with two units of RNase H at 37°C for 20 minutes.

6.2.1.3. Double-stranded cDNA synthesis: The cDNA obtained in previous step is amplified by PCR using the P1 and P3 primers and Go Taq® Green master mix (Promega). The PCR is carried out in a 100 µl reaction volume containing 5 µl of cDNA for 10-15

cycles. The PCR amplified DNA is visualized by running 5 µl of the reaction on an ethidium bromide stained 1.5% agarose gel. Usually, the band intensity is very low.

6.2.1.4. Cloning, sequencing and analysis of cDNA: The remaining PCR products (95 µl) are purified and concentrated to a volume of 15 µl using DNA Clean & Concentrator Kit (Zymo research). The purified PCR products (10 µl) are cloned into a low copy vector pWSK29 [25] using the restriction enzyme sites engineered into primers P1 and P3. At least 24 recombinant plasmids are sequenced using the primers complementary to the vector (either M13F or M13R) to determine the junction sequences between P3 and the cDNA. Any sequences following the three G nucleotides (last three nucleotides in P2) represent the 5' end of the cDNA. Recently, we have determined the RNase III cleavage sites and the mature 5' ends of 23S rRNA using this technique (Mohanty & Kushner, manuscript in preparation).

6.3. Analysis of 5' and 3' ends by self-ligation

The above two methods are limited to 5' end determinations. In fact, 3' end determinations have been more difficult. The 3' RACE method involving ligation of oligonucleotide to the 3' ends followed by RT-PCR cloning has been inefficient in our hands. Thus while working with tRNA processing and maturation, we felt the need for determining both the 5' and 3' ends of specific tRNAs. To carry out this type of experiment, we use a method involving self-ligation of 5' ends to 3' ends followed by RT-PCR cloning and sequencing of the junction of 5'-3' ends [26]. A detailed step by step of this procedure has described previously [22] and can be found in Appendix 4. The technique has been successfully used to determine both the 5' and 3' ends of *hisR*, *cysT*, *leuX*, *pheU*, *pheV*, *proK*, *proL*, *proM*, *leuU*, *secG* and 5S rRNA [5, 24, 27-30]. A sample analysis is shown in Fig. 4.

7. Concluding remarks

The significance of RNA metabolism in any biological system cannot be overstated. Until recently, RNA degradation in any biological system was viewed as a process designed to recycle ribonucleotides generated from mRNAs and noncoding RNAs that were no longer required for translation. With the discovery that small regulatory RNAs play a major role in the regulation of gene expression, the study of RNA processing and degradation has become increasingly important in helping to understand basic physiological processes in microorganisms. Thus, the identification and development of new techniques to study RNA metabolism is critical for understanding the mode of action of this work horse in biological system.

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Appendix 1:: Buffers and solutions

Tris-MGCl₂ (TM) buffer (5X):

50 mM Tris-base, 25mM MgCl₂, pH 7.2.

Stop Buffer (SB) (per 100 ml):

First, 50 mg chloramphenicol is dissolved in 12.5 ml of 95% ethanol. Subsequently, 20 ml of 5X TM buffer, 2.5 ml of sodium azide and 65 ml of water are added.

RNA extraction solution:

95% formamide (RNA grade, Sigma, 99.5% purity), 18 mM EDTA (pH 8), 0.025% SDS and 1% 2-mercaptoethanol

Tris-Acetate-EDTA (TAE) buffer:

40 mM Tris-acetate, 1 mM EDTA. The pH is usually around 8.6. Adjustment of the pH is not required.

TBE buffer (5X):

54 g Tris, 27.5 g boric acid, and 4.65 g EDTA. The pH is adjusted to ~8.0 and the total volume is made up to 1000 ml.

20X SSC (saline-sodium citrate):

3 M NaCl, 0.3 M sodium citrate. The pH is usually around 7.2. Adjustment of the pH is not required.

20% SDS:

200 g of SDS is dissolved in about 700 ml of water and the total volume is made up to 1000 ml.

Washing buffer:

20X SSC and 20% SDS is diluted to obtain a final concentration of 0.3% SDS and 2X SSC.

Appendix 2:: Northern analysis

Choosing an appropriate gel matrix for separation of RNA molecules for Northern analyses depends on the expected size of the transcripts. An acrylamide gel (5-10%) with 8.3 M urea is routinely used in our laboratory for separation of transcripts from less than 100 nt to less than 900 nt. An agarose Northern blot is suitable for the transcripts larger than 900 nt.

Acrylamide gel for Northern analyses:

We use a custom-made vertical gel apparatus for running acrylamide gels. However, any commercially available gel apparatus can also be used. The gel apparatus and the comb should be made RNase free by treating with RNaseZap® (Ambion®) before running the gel. An appropriate percentage of acrylamide gel is poured and allowed at least 2 hours for polymerization. The gel is assembled to the apparatus with 1X TBE (Appendix 1) as the running buffer. It is important to flush the loading wells with a syringe filled with 1X TBE

buffer to remove any unpolymerized acrylamide and urea. The acrylamide gel is pre-run at a constant current of 60 mA until the buffer temperature reaches ~55°C. All the loading wells are flushed again with 1X TBE buffer using the syringe before loading the gel.

Preparation of RNA samples for loading:

The RNA samples (5-10 µg) are dried in a Speedvac and resuspended in 5 µl of Gel Loading Buffer II (Ambion®). The sample is heated in a sand bath at 95°C for 5 minutes. The heated samples are briefly centrifuged to collect the liquids at the bottom before loading into gel while they are still warm. The gel is run at a constant current of 50 mA for an appropriate length of time.

Agarose gel for Northern analyses:

We routinely use glyoxal as denaturant for agarose Northern analyses. Agarose gels (1 or 1.5%) are run using horizontal gel apparatus as originally described by Burnett (1997) with following modifications. The dried RNA sample is resuspended in 5 µl of RNase free water and 5 µl of Glyoxal Load dye (Ambion®). The sample is heated at 50°C for 30 minutes in a water bath. The sample is chilled on ice for one minute and centrifuged briefly to collect the liquid at the bottom before loading onto the gel. The samples are separated at 5 volt/cm for an appropriate length of time.

Transferring RNA to a membrane:

We use electroblotting to transfer RNA from both the acrylamide and agarose gel to a positively charged nylon transfer membrane (0.45 µm, Nytran®SPC, GE Healthcare Life Sciences, Whatman™). The RNA is transferred in a cold room for 1 hr at 15 volts followed by 2 hrs at 40 volts. Larger transcripts (i.e. rRNAs) are transferred overnight at 15 volts. Following the transfer, the RNA is fixed to the membrane by baking at 80°C for 30 minutes followed by a 10 second exposure under an UV light. Although either one of the treatments is sufficient for fixing, baking has always works best for us.

Hybridization and washing of membrane:

We routinely use PerfectHyb™ Plus Hybridization Buffer (Sigma®) for both pre-hybridization and hybridization. Oligonucleotide probes are mostly used after 5' end labelling (Appendix 3). The membrane is pre-hybridized for at least one hour followed by overnight hybridization at appropriate temperature.

Subsequently, the membrane is washed three times (10 minutes each) with 100-200 ml of washing buffer (Appendix-1). The washed membrane is wrapped in Saran wrap for analysis by a PhosphorImager or exposure to X-ray film.

Appendix 3:: 5' end labelling of oligonucleotides

The end-labelling reaction is set up in a 10 µl reaction volume by adding 10-20 pmol primers, 1 µl 10X T4-PNK (polynucleotide kinase) buffer (provided by NEB), 5 µl γ -[³²P]-

ATP (6000 Ci mmol⁻¹, Perkin Elmer Life Sciences) and 10 units of PNK (New England Biolabs). After 30 minutes of incubation at 37°C, the reaction is inactivated at 95°C for 5 minutes. The end-labelled primer can be stored at 4°C until the next use for up to two weeks.

Appendix 4:: Analysis of 5' and 3' ends by self-ligation

In order to facilitate self-ligation of 5' and 3' ends, the 5' triphosphate end of a primary transcript is converted to 5' monophosphate by treating with either 5' Polyphosphatase (Epicentre®) or Cap-Clip™ Acid Pyrophosphatase (Cellscript™) following the manufacturer's instructions. The reaction is purified using phenol/chloroform extraction (section 4.1) followed by RNA precipitation (section 4.2). The RNA is resuspended in 4.5 µl of RNase-free water and heated at 65°C for 5 minutes, then the RNA is chilled on ice for one minute and centrifuged briefly to collect the liquid at the bottom of the tube.

The ligation reaction is carried out at 37°C for 2 hours using T4 RNA ligase 1 (NEB) according to manufacturer instructions. The ligation reaction is purified using phenol/chloroform extraction (section 4.1) followed by RNA precipitation (section 4.2). The RNA is then resuspended in 10.5 µl of RNase-free water and reverse transcribed using Superscript™ III reverse transcriptase (Thermo Fisher) as described in section 6.1.1.2. A transcript specific primer (usually 20-30 nt downstream of the expected 5' end) is used for this reverse transcription step.

The resulting cDNA is amplified using the RT primer at the 5' end and a second transcript specific primer at the 3' end using GoTaq® Green Master Mix (Promega). The PCR product is cloned into a low copy vector (pWSK29) [25] for sequencing. We use either a M13F or M13R primer whose complements are found in the vector to sequence the PCR insert.

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Highlights

1. This manuscript provides the reader with a comprehensive overview for analyzing RNA molecules isolated from either Gram-negative or Gram-positive bacteria.
2. All of the procedures are explained in detail either in the text or associated appendices.
3. The advantages and disadvantages of each procedure are discussed in some detail.
4. Examples of the data obtained using the procedures are provided.

Fig. 1. Steady-state RNA isolated from a wild-type and RNase III deletion (*rnc*) strains using the *RNAsnap*TM method. Total RNA (500 ng/lane) was separated on a 1% agarose gel and visualized with ethidium bromide staining. The various rRNA species are indicated to the right of the gel. The presence of the 30S primary rRNA transcript can easily be distinguished in the *rnc* mutant.

Fig. 2.

Analysis of 5' ends of *leuX* tRNA primary transcripts by primer extension [24]. All *E. coli* strains used in this study were derived from MG1693 (*rph-1*), which was considered wild-type. MG1693 has a single base pair deletion in the *rph* gene resulting in a frame-shift mutation [31]. The genotypes of the strains are noted at the top of the lane. Total RNA isolated from all the strains were reverse transcribed using a ^{32}P -end labelled *leuX* tRNA specific primer and the cDNAs were separated on a 6% polyacrylamide sequencing gel. A *leuX* DNA sequencing ladder (CTAG) generated by same *leuX* tRNA specific primer was also separated along with the cDNAs and was used to identify the transcription initiation site (I) and the mature 5' end (II). The primer extension analysis also identified several RNase E cleavage sites (indicated as *) in the *rnpA49* mutant, which disappeared in the *rne-1 rnpA49* strain.

Fig. 3. Graphical presentation of SMART™ cloning. P1, gene specific RT primer; P2, SMART template switching primer and P3, anchor primer. The procedure is as described in the text (section 6.2)

Fig. 4.

Analysis of *proK* tRNA 5' and 3' end processing in an RNase P mutant using the self-ligation RT-PCR cloning technique. The cartoon at the top represents a graphical presentation of genomic organization of the monocistronic *proK* gene. The transcription initiation sites (underlined) upstream of the mature *proK* tRNA (rectangle) and the downstream immature sequences with the transcription terminator are shown [32]. The downward arrow ($\downarrow$) represents the positions of either a 5' or a 3' end based on sequencing of the cDNAs. The numbers of 5' and 3' ends detected were noted on top of the arrow. The number of transcripts with unprocessed 5' ends increased significantly in the *rnpA49* mutant compared to the wild-type control [32]. Both strains also contain *rph-1* allele (see legend to Figure 2.)