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Short Communication

An effective method for obtaining high quality messenger ribonucleic acid (mRNA) with minimal ribosomal ribonucleic acid (rRNA) contents

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Plant transcriptomes are very complex in nature and includes overlapping transcripts, transcribed intergenic regions, and abundant non-coding ribonucleic acids (RNAs). With RNA-Seq approach using next generation sequencing technology, complementary deoxyribonucleic acid (cDNA) library constructed either from Messenger ribonucleic acid (mRNA) or from ribosomal ribonucleic acid (rRNA) minus total RNA can be directly sequenced for transcriptome studies. The transcriptome analysis is mainly hampered by presence of unwanted abundant rRNA transcripts in cDNA library, which may represent majority species in RNA-Seq if not removed carefully from the total RNA. Though many commercial kits are available in the market for isolation of high quality mRNA from total RNA and their efficiency to remove rRNA contaminants from mRNA may vary. In the present study an effort has been made to isolate high quality mRNA with minimal rRNA contamination through designing an experiment with the use of two commercially available kits.

Key word: RNA-Seq, transcriptome, next generation sequencing, ribosomal RNA, bioanalyzer.

INTRODUCTION

The recent advances in high-throughput sequencing technologies revolutionized the way to analyze complex transcriptomes. In the present scenario of next generation sequencing, RNA-Seq is perhaps one of the most complex next generation applications (Costa et al., 2010), which yields a comprehensive view of both the transcriptional structure and the expression levels of transcripts (Nagalakshmi et al., 2010). Transcriptome analysis includes identification of each transcribed regions, overlapping transcripts, transcribed intergenic regions and abundant non-coding RNAs present in a cell or tissue and quantification of its transcriptional level under specific biological condition. Variation in transcriptome is related to the variation in cell, tissue and even in the lower to higher organism (Varsale et al., 2010).

In transcriptome analysis, high quality mRNA is a prerequisite for constructing cDNA library. Since large ribosomal RNA (rRNA) constitutes 80 to 90% RNA species in total RNA (Lindberg and Lundberg, 2010), the transcriptome analysis is mainly hampered by presence of unwanted abundant rRNA transcripts in cDNA library, which may represent majority species in RNA-Seq if not removed carefully from total RNA. Though many

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Abbreviations: RNAs, ribonucleic acids; **rRNA,** ribosomal ribonucleic acid; **mRNA,** messenger ribonucleic acid; **cDNA,** complementary deoxyribonucleic acid; **tRNA,** transfer ribonucleic acid; **miRNA,** micro ribonucleic acid; **siRNA,** short interfering ribonucleic acid; **ncRNA,** non coding ribonucleic acid.

Table 1. Quantification of RNA using nanodrop spectrophotometer.

Sample	Concentration (ng/µl)	260/280 ratio	Volume (µl)	Final yield (µg)
Total RNA	600	1.9	160	96.0
mRNA	106	1.9	20	2.1
Ribo-minus mRNA	36	2.1	20	0.7
Ribo-minus Total RNA	122	2.0	20	2.4

commercial kits are available in the market for isolation of high quality mRNA from total RNA and their efficiency to remove rRNA contaminants from mRNA may vary. The present study was initiated due to unexpected results of our previous experiment in which a cDNA library was constructed from mRNA using cDNA Synthesis System Kit (Roche) and Rapid Library Prepration Kit (Roche) as per cDNA Rapid Library Preparation Method Manual (Roche) and sequenced on Roche GS FLX Titanium. The final data generated showed 80 to 85% rRNA sequences and only 15 to 20% was of our interest (mRNA sequences). Therefore, an effort has been made to isolate high quality mRNA with minimal rRNA contamination through designing an experiment with the use of two commercially available kits.

MATERIALS AND METHODS

Fresh plant leaves of *Ocimum sanctum* (commonly known as 'Tulsi') were taken as the source of total RNA. Total RNA from the leaves stored in RNA *later*® (Applied Biosystems now is life technologies) solution was extracted from 100 mg of sample grind in liquid nitrogen in replicates using Trizol® reagent methods (Invitrogen, Cat. No. 15596-026) followed by *DNase*I enzyme treatment to remove DNA contamination. To purify mRNA from isolated total RNA, we followed three different approaches. In the first approach, mRNA was purified using a commercial mRNA isolation kit containing biotinylated oligo $(dT)_{20}$ probes (Roche, Cat. No. 11741985001) following the manufacturer's protocol. In the second approach another commercial RiboMinus™ Plant Kit from Invitrogen (Cat No. A10838-02) was used. This kit includes 6 probes each with specificity for plant nuclear (25/26S, 17/18S), chloroplast (23S, 16S), and mitochondrial (18S) rRNA. The Ribo-minus RNA was concentrated by using RiboMinus™ Concentration Module (Invitrogen, Cat. No. K1550-05). The third approach combines the use of two kits and involves isolation of mRNA from total RNA through oligo $(dT)_{20}$ probes using Roche mRNA isolation kit followed by its further purification by using RiboMinus™ Plant Kit. RNA concentration was determined by measuring the absorbance at 260 nm (A260) on nanodrop spectrophotometer (Thermo Scientific, USA). The purity and integrity of RNA is critical to the success of any RNA-based analysis, therefore, purity and integrity of total RNA, mRNA, Ribo-minus total RNA and Ribo-minus mRNA samples were checked using Agilent 2100 Bioanalyzer with RNA 6000 pico lab chip.

RESULTS AND DISCUSSION

The concentration of total RNA as determined by measuring the absorbance at 260 nm (A260) with nanodrop was observed as 600 ng/µl with a ratio of 1.9 (Table 1). Integrity of total RNA was checked in replicates using Agilent 2100 Bioanalyzer with RNA 6000 pico lab chip. The instrument allows quantitative and qualitative analysis of the samples by capillary electrophoresis and provides a platform to record the size distribution of molecules like RNA in a digital format (Schroeder et al., 2006). The produced value called RIN (RNA Integrity Number) allows assignment of a qualitative index to the analyzed RNA. This parameter forms a scale of 0 to 10 where the value10 stands for RNA of excellent conditions and zero stands for totally degraded RNA. The RIN includes not only the peaks representing rRNA fragments, but also the background and the intensity of possible degradation products (Vennemann and Koppelkamm, 2010). The efficiency of rRNA removal from mRNA, Ribo-minus total RNA and Ribo-minus mRNA, RNA degradation and RNA concentration was also analyzed using Agilent Bioanalyzer and results were compared (Figure 1). In case of total RNA the RIN value was observed to be 6.9, which was good enough to proceed further (Figure 1a). In case of mRNA isolated from total RNA, we found about 10.0% rRNA contamination mainly due to the presence of 18S and 28S rRNA (Figure1b). From 96 µg of total RNA, we got 2.1 µg of mRNA.

In case of Ribo-minus total RNA, we found about 2.4% rRNA contamination (Figure 1c) and the yield of Ribo-minus total RNA isolated from 10 µg total RNA was 2.4 µg. Compared to the mRNA fraction, the Ribo-minus total RNA fraction is enriched in polyadenylated (polyA) mRNA along with nonpolyadenylated RNA, pre-processed RNA, Transfer ribonucleic acid (tRNA) and may also contain regulatory RNA molecules such as micro ribonucleic acid (miRNA), short interfering ribonucleic acid (siRNA), small Non coding ribonucleic acid (ncRNA) and other RNA transcripts (Jacquier et al., 2009). In case of Ribo-minus mRNA the rRNA contamination was reduced to 1.1% along with enrichment of mRNA region (Figure 1d). For isolation of Ribo-minus mRNA, we used 2.1 µg of mRNA which yield about 0.7 µg of Ribo-minus mRNA. The quality of mRNA as we got by Ribo-minus mRNA procedure was comparatively good along with minimal rRNA contamination. However, this procedure requires an approximately double amount of total RNA (due to less recovery of mRNA after Ribo-minus of mRNA) as compared to other approaches.

In our conclusion, the ribo-minus of mRNA approach seems to be an effective method for isolation of high

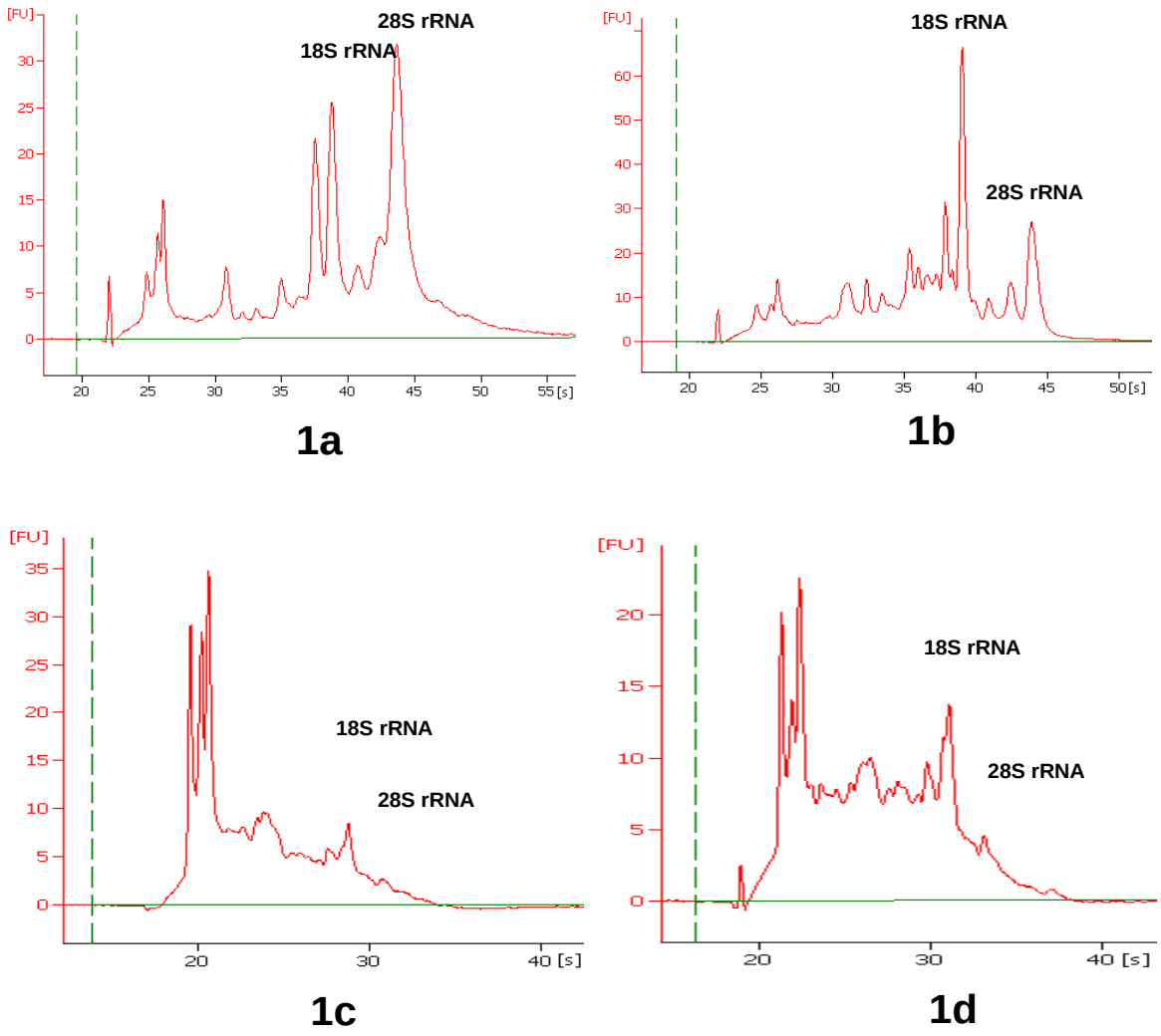

Figure 1a. Total RNA profile with RIN 6.9 showing 18S rRNA and 28S rRNA peaks. **b.** mRNA isolated from total RNA profile showing 18S rRNA and 28S rRNA peaks. **c.** Ribo-minus total RNA profile (total RNA → Ribo-minus). **d**. Ribo-minus mRNA profile (total RNA → mRNA → Ribo-minus).

quality mRNA with minimal rRNA contamination and we hope that final sequencing data generated from Ribo-minus mRNA will be of good quality having majority of mRNA sequences.

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