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Note

A robust and efficient method for the isolation of DNA-free, pure and intact RNA from *Mycobacterium tuberculosis*

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ABSTRACT

We describe a robust method for the isolation of pure, intact RNA suitable for transcriptome studies from mycobacteria with consistent yields of 1 μg to 3 μg total RNA per 10^7 cells. The method reduces the use of hazardous chemicals and incorporates protocols for efficient removal of gDNA and rRNA.

Microarrays, real time PCR and RNA sequencing are powerful technologies to quantify mRNA for understanding physiological events at the whole genome-level (Berry et al., 2010; Caoili et al., 2006; Stewart et al., 2002). For the success of these RNA based analysis, purity and integrity of input RNA are critical factors (Copois et al., 2007; Nygaard et al., 2003; Vermeulen et al., 2011), and any compromise in RNA quality can lead to variable results (Imbeaud et al., 2005; Raeymaekers, 1993). Hence, it is important to develop new methods for RNA extraction from cells and tissues, which can reproducibly yield RNA of high quality and purity.

Degradation of RNA can occur at any step of isolation or during storage under sub-optimal conditions (Perez-Novo et al., 2005). To minimize this, the extraction protocol should be rapid with minimal transfer steps and should effectively eliminate RNase activity. Fragmentation of chromosomal DNA and its contamination in RNA preparations are a common source of error in the quantification of mRNA levels (Phongsisay et al., 2007). This is especially true for the quantification of mRNA from bacteria wherein the mRNA and genomic DNA (gDNA) have the same genetic presentation and are therefore indistinguishable. Hence, the elimination of gDNA contamination from RNA preparations is important for expression profiling studies.

The RNA extraction process becomes considerably difficult in thick-walled bacteria such as *Mycobacterium tuberculosis*, which are resistant to quick and efficient lysis. Several methods have been reported for the isolation of RNA from mycobacteria (Cheung et al., 1994; Dietrich et al., 2000; Payton and Pinter, 1999) including the Trizol-based method and the Hot-phenol based method (Barry et al., 1992; Mangan et al., 1997; Monahan et al., 2001; Rajagopalan et al., 1995; Rio et al., 2010; Rustad et al., 2009). However, these methods use highly toxic chemicals in several steps and give variable quality of RNA (Jahn et al., 2008).

Herein, we describe a reproducible method for the preparation of gDNA-free, pure and intact RNA from mycobacteria. The RNA isolated by this method conforms to the standards documented for RNA quality (Imbeaud et al., 2005) and is suitable for transcriptomic studies.

M. tuberculosis H_{37}Rv and *M. smegmatis* mc^2155 were grown in Middlebrook 7H9 (Difco, Becton Dickinson and Co., USA) supplemented with 0.05% Tween 80 and 10% Albumin, Dextrose, Catalase (Difco, Becton Dickinson and Co., USA). *E. coli* DH5α was grown in Luria Bertani medium. All cultures were grown at 37 °C with continuous shaking at 200 rpm to A_{600nm} of ~ 0.6–0.8.

For isolation of RNA, the cells were harvested from a 25 ml culture of *M. tuberculosis*, *M. smegmatis* or *E. coli* by centrifugation at 2500 *g* for 10 min. Each method was performed in two separate sets of experiments in triplicates. Method 1 involved isolation of RNA using Trizol reagent (Rustad et al., 2009). Briefly, cell pellet was suspended in 1 ml of Trizol reagent (Life Technologies Corporation, USA) and subjected to bead beating for 10 pulses of 30 s each in a bead beater (Biospec Inc. USA) after addition of 0.4 ml of glass beads (600 μm acid-washed beads, Sigma-Aldrich, USA). The sample was cooled on ice for 30 s between successive bead beating cycles. The resulting cell lysate was centrifuged at 12,000*g*, 4 °C for 10 min and the aqueous phase was extracted with chloroform and precipitated with chilled isopropanol. The RNA pellet obtained was washed with 75% ethanol, air-dried and suspended in 50 μl RNase-free water.

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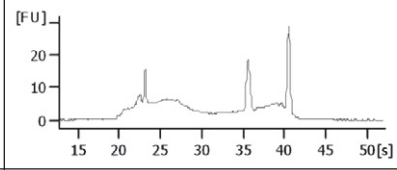
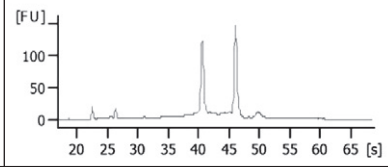
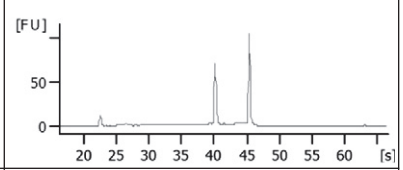
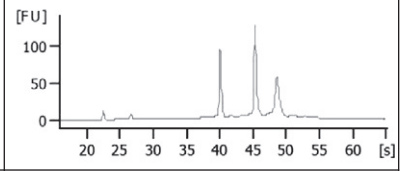
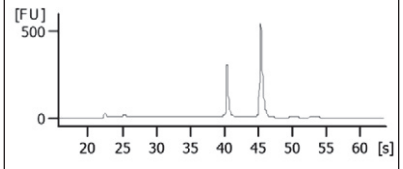
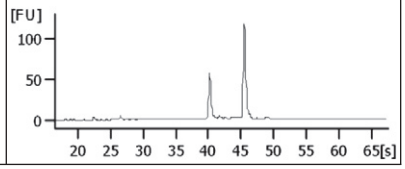
Method of RNA isolation	Organism	Volume of culture	RNA Yield	Photo of Agarose Gel	A_{260nm}/A_{280nm}	A_{260nm}/A_{230nm}	23S /16S rRNA	RIN	Bioanalyser Chromatogram
Trizol (Method 1)	Mtb	25 ml	40 µg		1.63	2.17	NA	5.1	
Hot Phenol (Method 2)	Mtb	25 ml	39 µg		1.72	1.17	1.3	8.6	
Modified Rneasy (Method 3)	Mtb	25 ml	54 µg		1.94	2.17	1.3	9.8	
Modified RNeasy	Msm	25 ml	100 µg		2.04	2.02	1.4	9.1	
Modified RNeasy	*E. coli*	25 ml	25 µg		2.07	2.00	2.3	9.7	
Modified RNeasy	Mtb	3 ml; A_{600}- 0.2	3.6 µg		2.20	1.90	1.9	9.7	

Fig. 1. Analysis of RNA samples isolated by different methods. Mtb, *M. tuberculosis*; Msm, *M. smegmatis*.

Method 2 involved extraction using Hot-Phenol (Kumar et al., 2009). In this, the cell pellet was suspended in 400 µl warm TES buffer (10 mM Tris–HCl pH 7.5; 10 mM EDTA pH 8.0 containing 0.5% SDS). Acid-washed glass beads (0.4 ml) were then added to the cell suspension followed by the addition of 0.4 ml of hot water-saturated phenol. The contents were vortexed vigorously for 2 min followed by incubation at 65 °C for 1 h with intermittent vortexing every 15 min. After centrifugation, the aqueous layer was extracted twice with chloroform:isoamylalcohol (24:1) followed by precipitation with ethanol. The RNA pellet obtained was washed with 70% ethanol, air dried and suspended in 50 µl RNase free water.

Method 3 is a newly optimized modified RNeasy method for RNA isolation. It involves enhanced cell lysis, RNeasy spin column purification and two-step DNase digestion. In this method, the cell pellet was suspended in 100 µl of TE buffer (10 mM Tris pH 7.5, 1 mM EDTA pH 8.0) containing 20 mg/ml lysozyme (USB Corp., USA) and 10 µl Proteinase K (1 U/µl; Qiagen Inc., USA). The suspension was vortexed, and incubated at room temperature for 10 min with intermittent vortexing every 2 min. 700 µl of RLT buffer (from RNeasy kit, Qiagen Inc.) was added to the suspension followed by mixing. The suspension was transferred to 1.5 ml screw cap vials containing 0.4 ml of acid-washed glass beads and subjected to bead beating for 10 pulses of 20 s each with intermittent chilling on ice. After centrifugation at 12,000 *g* for 20 s at room temperature, the supernatant was transferred to a fresh tube, mixed with 590 µl of 80% ethanol and transferred to RNeasy spin column. The column was then spun at 12,000*g* for 15 s. 350 µl of RW1 buffer was added to the column and the column was centrifuged for 15 s at 12,000*g*. A total of 10 µl of RNase free DNase diluted in 70 µl of RDD buffer was added to the column and incubated at room temperature for on-column DNase digestion to remove contaminating genomic DNA (gDNA). After 15 min, 350 µl of RW1 buffer was added, the column was centrifuged for 15 s at 12,000*g*, washed with RPE buffer twice and the RNA was eluted in 50 µl of RNase-free water. These RNA samples were subjected to a second step of gDNA decontamination by in-solution DNase treatment using DNA-Free (Ambion, Life Technologies Corporation, USA) as per manufacturer's instructions.

The absorbance of the samples was measured by using the Nanodrop 2000c spectrophotometer (Thermo Scientific, USA). Yield of the RNA was calculated from the absorbance of the sample at 260 nm. Purity and integrity of RNA were assessed by microfluidics-based capillary electrophoresis