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An Effective Method of RNA Extraction from *Mycobacterium tuberculosis*

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In the RNA-based study, it is important to extract high-quality RNA. However, RNA extraction from *Mycobacterium tuberculosis* is problematic due to its thick, waxy cell wall rich in mycolic acid, which renders the cells resistant to lysis. Using TRIzol reagent and several powerful bead-beating steps, a high quantity of RNA was obtained. **(Ann Clin Microbiol 2016; 19:20-23)**

Key Words: *Mycobacterium tuberculosis*, RNA extraction, Quantitative reverse transcription polymerase chain reaction

Tuberculosis due to infection with *Mycobacterium tuberculosis* is one of the most important communicable diseases [1]. Because *M. tuberculosis* grows more slowly than most other bacteria, direct analysis of RNA expression is a major area of interest [2]. To successfully quantify the expression of specific *M. tuberculosis* genes by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) or RNA sequencing (RNA-Seq), it is important to use intact and pure RNA that is free of DNA and proteins as a template [3]. However, RNA extraction from *M. tuberculosis* is problematic due to its thick, waxy cell wall rich in mycolic acid, which renders the cells resistant to lysis [4]. Furthermore, because RNA is subject to degradation during any extraction or purification steps [5], sophisticated handling techniques under optimal conditions are necessary.

Various methods for extraction of mycobacterial RNA have been reported based on enzymatic hydrolysis, chemical treatment, french pressure cell rupture, bead-beating or sonication [2,4]. We first tried to extract RNA based on enzymatic hydrolysis with lysozyme and RNeasy Protect Bacteria Mini Kit (Qiagen, Venlo, Netherlands) for purify RNA but it was problematic. Larsen et al. introduced two methods for extraction of RNA from *M. tuberculosis*: the RNA-TRIzol protocol and the Fast Prep method [6]. Each protocol of Larsen et al. used TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) or bead beater individually. We modified the above methods for our laboratory and successfully extracted intact and pure RNA. Here, we describe our simple RNA extraction method that involves simultaneous use of TRIzol reagent (Thermo Fisher Scientific) and a bead beater in a single extraction step (Table 1). To avoid RNA degradation, samples should be handled quickly and be kept on ice at all times, except during reactions with reagents.

Fifteen multidrug-resistant *M. tuberculosis* strains, fifteen extensively drug-resistant *M. tuberculosis* strains, and *M. tuberculosis* H37Rv were cultured in 30 mL Middlebrook 7H9 broth (BD, Franklin Lakes, NJ, USA) supplemented with 10% oleic acid, albumin, dextrose, catalase enrichment (BD), and 0.5% (v/v) glycerol in a 37°C incubator for 2-3 weeks. An aliquot (7 mL) of culture was then transferred to 15 mL screw-cap tubes and centrifuged at 2,200 g and 4°C for 10 min with removal of supernatant to harvest mycobacterial cells, which were washed in 1 mL Tris-EDTA buffer (pH 7.5) by tapping tube to remove excess 7H9 broth, centrifuged again using identical conditions, then resuspended in 1 mL RNAprotect Bacteria Reagent (Qiagen)

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Table 1. Flow of RNA extraction of *M. tuberculosis*

Phase	Time
Sample homogenizing	
Cell harvest	35 min
Spin (7 mL of bacterial cells)	10 min
Decant of supernatant and wash with Tris buffer, Spin	10 min
Decant of supernatant and incubation with RNA protect bacteria reagent	5 min
Spin	10 min
Cell disruption	43.5 min
Bead beating with Trizol solution and incubation (repeat four times)	23 min
Shaking with chloroform	0.5 min
Incubation	5 min
Spin	15 min
RNA isolation	
RNA precipitation	25 min
RNA precipitation with isopropyl alcohol	20 min
Spin	5 min
RNA wash and resuspension	5 min
Decant of supernatant and wash with ethyl alcohol	Briefly
Spin	5 min
Air dry	Briefly
Dissolve with DEPC-treated water	
DNA digestion	
Activation of DNase	60 min
Incubation with TURBO DNase buffer and TURBO DNase	60 min
Inactivation of DNase	6.5 min
Incubation with DNase inactivation reagent	5 min
Spin and transfer RNA of supernatant to new tube	1.5 min

by vortex-mixing. The cells were then incubated for 5 min at room temperature and centrifuged again. After removal of the supernatant, the pellet can be preserved for several months at −80°C or proceed to the next step. The pellet was resuspended in 1 mL TRIzol reagent (Thermo Fisher Scientific), transferred to a Lysing Matrix B tube (MP Biomedicals, Santa Ana, CA, USA) containing 0.1 mm silica beads, and processed four times using a Fast Prep-24 5G (MP Biomedicals) instrument for 45 s using a speed setting of 6.5. After each bead-beating run, the tube was incubated in an ice block, and at room temperature in the last run. Chloroform (0.2 mL) was added to lysates and tubes were shaken manually for 30 s. Lysates were incubated for 5 min at 4°C and centrifuged at 13,400 g and 4°C for 15 min. The aqueous upper layer of chloroform containing RNA was transferred to a new 1.5 mL tube and RNA was precipitated by adding 0.5 mL isopropyl alcohol, followed by incubation for 20 min at −80°C. The precipitated RNA was centrifuged at 13,400 g and 4°C for 10 min and the supernatant was decanted. The RNA pellet was washed by brief vortexing, mixing with 1 mL 75% ethanol, re-centrifuged at 13,400 g and 4°C for 5 min, air-dried briefly, and dissolved in 50 μL diethylpyrocarbonate (DEPC)-treated water. RNA concentration and purity (A260/A280 nm) were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and electrophoresis in a 1% agarose gel followed by ethidium bromide staining. To eliminate genomic DNA contamination, DNA was digested using a TURBO DNA-free Kit (Thermo Fisher Scientific). RNA was diluted with DEPC-treated water to 250 ng/μL and treated with 6 U of DNase (the maximum recommended by the manufacturer) at 37°C for 1 h. The measured concentration of extracted RNA treated with DNase were ~126-208 ng/μL and purity (A260/A280 nm) of that were 1.66-1.84, respectively. Two clear bands of 16S and 23S rRNA were identified on the gel (Fig. 1). PCR amplification of *rpoB* (housekeeping gene) was performed to check for genomic DNA contamination. The forward and reverse primers used were newly designed by Bioneer (Daejeon, Korea), and their sequences are as follows: 5′-GTTCAAGG TGCTGCTCAAAG-3′ and 5′-GGACAGATTGATTCCCAGGT-3′, respectively. The annealing temperature was 59°C. Then, amplified products were validated free of genomic DNA by electro-

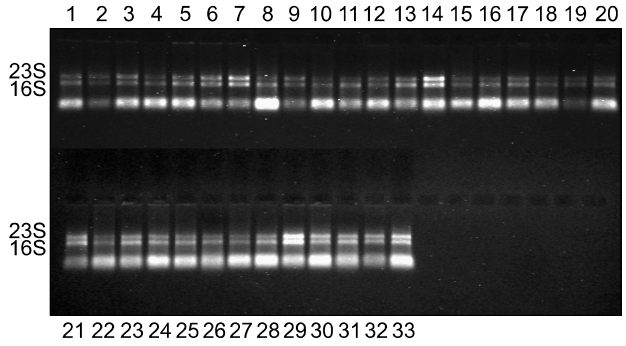

Fig. 1. Ethidium bromide-stained 1% agarose gel of RNA extracted from clinical isolates of *M. tuberculosis*. All lanes showed 16S and 23S rRNA bands. Lanes 1 to 33: *M. tuberculosis* clinical isolates; lanes 25, 26 and 27 were identical isolates with lanes 19, 23 and 24 respectively.

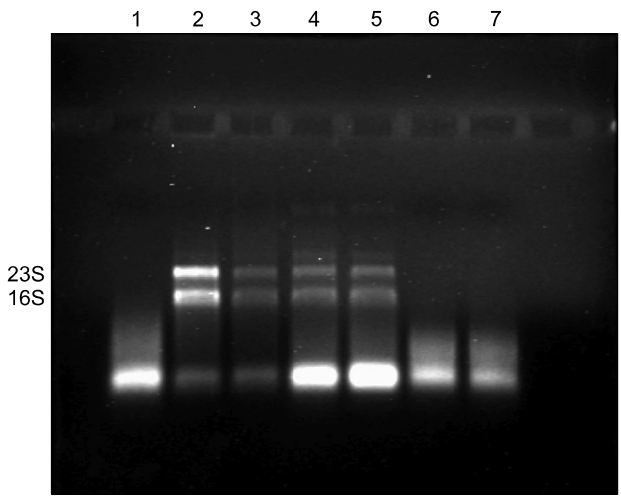

Fig. 2. Ethidium bromide-stained 1% agarose gel of RNA extracted from *M. tuberculosis* clinical isolates and strain H37Rv. Lanes 2 to 5: 16S and 23S rRNA bands. Lane 1: *M. tuberculosis* clinical isolate grown in MGIT; Lane 2: *M. tuberculosis* H37Rv cultured in Enriched Middlebrook 7H9 Broth; Lanes 3 to 5: *M. tuberculosis* clinical isolates cultured in Enriched Middlebrook 7H9 Broth; Lanes 6 to 7: *M. tuberculosis* clinical isolates cultured in MGIT.

phoresis in a 2% agarose gel and ethidium bromide staining (Fig. 1).

For RNA extraction, we compared two *M. tuberculosis* growth media: Enriched Middlebrook 7H9 broth and Mycobacteria Growth Indicator Tube (MGIT) (BD). Seven milliliters of bacterial culture were used to extract total RNA using the extraction method described above. However, RNA extracted from MGIT cultures showed low yield or degradation (Fig. 2), in contrast to previous report [7]. Two culture media are almost same except casein peptone and fluorescence indicator. However, we inoculated a loopful of bacteria in media directly, the rate of proliferation could be faster than usual. The volume (7 mL) and thin tube of MGIT could be insufficient for the growth of bacteria for 3 weeks. Because the volume and container of 7H9 broth we used were 30 mL and Erlenmeyer flask, environment for growth of mycobacteria could be more ideal than that of MGIT. Also, we investigated whether the diameter of the beads used for bacterial cell lysis could affect the yield and/or quality of RNA. We extracted RNA from *M. tuberculosis* using beads of diameters 0.1 mm, 0.2 mm and 0.2-0.5 mm mixture; the results did not differ significantly among the three bead diameters. In the early stage of this study, RNA was purified using an RNeasy Protect Bacteria Mini Kit (Qiagen); however, this led to a loss of over 50% of total RNA after cleanup. It is believed that the RNA could not adhere to the surface of the column during purification. To reduce RNA loss, we performed DNase treatment after precipitation of RNA without column cleanup; this reduced RNA loss to ~20%. Thus, if genomic DNA is not identified by gel electrophoresis and the RNA is of reasonable purity, additional purification is unnecessary.

Extraction of intact and pure RNA is important but problematic. Using TRIzol reagent and several powerful bead-beating steps, a high quantity of RNA was obtained. After treatment with a high concentration of DNase, the extracted RNA is available for use in subsequent experiments.

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=국문초록=

*Mycobacterium tuberculosis*의 효과적인 RNA 추출방법

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RNA 기반 연구에서 고품질의 RNA 추출은 상당히 중요하다. *Mycobacterium tuberculosis*는 세포벽에 mycolic acid가 풍부하여 두껍고 왁스와 같은 성질을 띈다. 이로 인해 용균이 쉽지 않아 RNA 추출이 어렵다. TRIzol 시약과 silica bead를 이용한 균 파쇄를 통하여 높은 수율의 RNA가 추출이 가능하였다. [Ann Clin Microbiol 2016;19:20-23]

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