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Full Length Research Paper

Validation of RNA integrity from low yield experiments with *Mycobacterium tuberculosis* for downstream application in real time PCR

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RNA extraction from mycobacterial cells is more challenging than from any other cell type. We used the Trizol Reagent® with silica beads to disrupt cells of *Mycobacterium tuberculosis* H37Rv. This method requires minimum reagents and handling and therefore aids in maintaining RNA integrity. The efficiency of cell disruption by this method was verified by microscopic analysis of the lysate. The yield and purity were determined using the Nanodrop-1000. A total volume of 50 µl containing 224.7 ng/µL of RNA was obtained from 3.1 x 10^{8} cells, grown under sub-optimal conditions. RNA was visualized using the gene genius optical system. A 6 week old undisturbed culture of *M. tuberculosis* H37Rv, gave an expression ratio of *fdxA*/16s of -2.094. This extraction method is suitable for use of RNA in quantitative experiments, even if the cell numbers from which the RNA is extracted are low.

Key words: Mycobacteria, RNA extraction, RNA purification, quantitation.

INTRODUCTION

Despite existing knowledge and treatment modalities, infectious diseases like tuberculosis and viral hemorrhagic fevers still pose a threat to mankind (Dye C et al., 1999). There is an urgent need to gain a thorough understanding into the physiology and survival mechanisms of such organisms. Such understanding will largely be obtained through a genomic approach. High quality nucleic acid is essential for this type of research. The robust and complicated cell wall structure of mycobacteria renders RNA extraction from these bacteria more difficult when compared to other cells (Brennan and Nikaido, 1995; Daffe etal., 1993). Real time PCR has widespread application in targeted gene expression analysis. Gene expression studies can be conducted by using primers and probes to directly detect messenger RNA transcripts (Wacker and Godard, 2005; Bustin and Nolen, 2004). The amount of mRNA produced reflects the level of protein production coded for by the gene that forms the template for that mRNA. It is however common practice to obtain total RNA which includes the ribosomal RNA and the mRNA of other housekeeping genes. These transcripts may be employed for normalization of gene expression. Total RNA extraction followed by reverse transcription into cDNA allows for multiple gene analysis on the same transcription product (Wacker and Godard, 2005; Bustin, 2002).

RNA extraction is a meticulous process, in which the slightest error in technique can adversely affect both the quality and quantity of the final product. The Trizol Reagent (Invitrogen, Life Technologies) contains a patented combination of stabilizers that prevents RNA degradation. The manufacturer describes extraction methods for most cell types. In the case of bacteria, it specifies use on gram positive and gram negative cells, but not acid fast bacteria. Due to their robust cell wall structure, an additional cell disruption mechanism is required for mycobacteria in order to obtain reasonable yields of RNA (Mangan et al., 1997). The yield of RNA is further affected by the clumping of some species of myco-bacteria (Zhang et al., 1998). While sonication is an effective disruption method for mycobacteria, it is known to affect the expression of the gene transcripts and this method is therefore not recommended (Timm et al., 1999).

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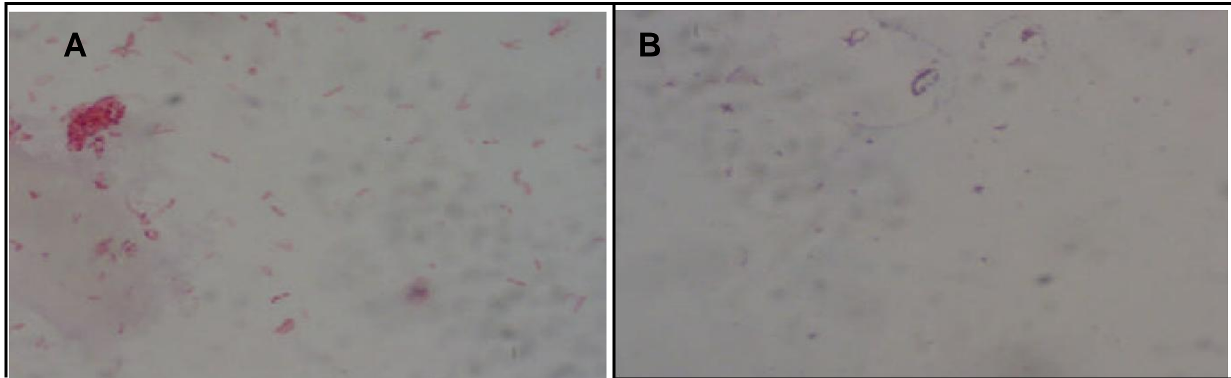


Figure 1. Ziehl-Neelsen Stain showing the presence of intact cells (A) and cells that have been completely disrupted (B).

Mechanical disruption by means of mini-bead beaters is commonly used (Mangan et al., 1997; Stephen et al., 2004) as this disrupts the bacterial clumps and breaks the wall of mycobacterial cells. We describe a simplified method that combines the physical and mechanical disruption of mycobacterial cells.

MATERIALS AND METHODS

A 6 week old standing broth culture of the laboratory strain H37Rv was used to test this method at a cell density of A600 = 0.961. Each step was done at 4°C unless stated otherwise. 10 ml of a broth culture was centrifuged at 4500 x g for 25 min. The supernatant was removed by aspiration and 0.5 ml Trizol Reagent ® was added to the pellet. The bacteria were resuspended by repeated pippetting till the mixture appeared homogenous. The suspension was transferred to a pre-chilled sterile nuclease free micro-centrifuge tube containing 300 g silica beads (Sigma). The Ziehl Neelsen stain was used to validate the cell disruption method described below. All slides were prepared in biosafety cabinets. Briefly, 100 µl of cell lysate was applied to microscopy slides and fixed by dry heat at 70°C for 2 h. Slides were covered in 0.3% carbol fuchsin for 5 min. A flame was used beneath the slides to heat evenly until vapours are seen. The slides were then rinsed with distilled water. A solution of 3% HCl in 96% ethanol was applied for 2 min to de-colorize non-acid fast material. The efficacy of this process is illustrated in Figure 1. The slides were then rinsed with water and counterstained for 2 min With 1% methylene blue. After rinsing, the slides were dried and viewed at 1000 x magnification.

The suspensions were vortexed 5 times for 30 s at maximum speed. After incubation at room temperature for 5 min, 0.1 ml cold chloroform (4°C) was added and mixed with the cell lysate by inversion. After a further incubation at room temperature for 3 min, the chloroform phase was separated from the water phase by centrifugation at 12,000 x g for 15 min. The upper phase was transferred to a pre-chilled sterile nuclease free microcentrifuge tube.

The DNA was removed by adding 2 l (1U/ l) DNAse 1 (Fermentas Life Sciences) and 2 l 10 X Reaction buffer with $MgCl_2$ (Fermentas Life Sciences). This mixture was placed in a heating block at 37°C for 30 min. To inactivate the enzyme, 2 l of 25 mM EDTA solution (Fermentas Life Sciences) was added followed by incubation for 15 min at 65°C. To precipitate RNA, the solution was mixed by inversion with 0.4 ml cold isopropanol and incubated at room temperature for 10 min. The tubes were centrifuged at 15,000 x g for 10 min and the supernatant was removed without disturbing the RNA pellet. The pellet was washed in 0.8 ml 75% ethanol and repelleted by centrifugation at 7,500 x g for 5 min. After aspiration of ethanol, the RNA pellet was air -dried. The RNA was resuspended in 50 l DEPC treated water and dissolved by heating at 60°C for 10 min. The importance of this is exemplified in Figure 2.

To further validate the method, 2 g of total RNA was converted to cDNA (High Capacity cDNA Archive Kit, Applied Biosystems). Most methodologies establish the presence of the RNA subunits by electrophoresis in a gel (Mangan et al., 1997, Stephen et al., 2004). This requires large quanities of RNA. The Syngene automatic imaging system uses optical viewing technology to detect the presence of electrophoresed products in agarose gels (http://www.syngene.com/html/software.html). This method allows detection of smaller quantities of nucleic acids (Figure 3) . It offers the added advantage of detecting the presence of the electrophoresed ribosomal RNA fragments even in the event of obscurity to the naked eye. The expression of the ferredoxin coding gene fdxA (Rv2007c) was quantitated against the 16S ribosomal RNA as an endogenous control. The default number of cycles of the1 ABI 7000 sequence detection system was used for all real-time PCR experiments.

Further, the examination of gene expression directly in clinical specimens is important in mycobacteriology research. Desjardin et al. (1996) showed that NALC-NaOH decontamination of clinical specimens results in damage to RNA. The method described allows for examination of gene transcripts when conducting experiments on cells that result in retarded cellular growth and therefore in poor RNA yields. This type of application includes, drug testing, nutrient and oxygen depletion studies, etc. This method therefore has potential application with necessary modification for *in vivo* research and is therefore a valuable research tool when low RNA yields are anticipated.

RESULTS AND DISCUSSION

The Ziehl-Neelsen stain was used to determine the effectiveness of this method of cell disruption. Vortexing in Trizol Reagent® with silica beads (Ø 150 - 212 m) was shown to be effective in disrupting mycobacteria as illustrated in Figure 1.

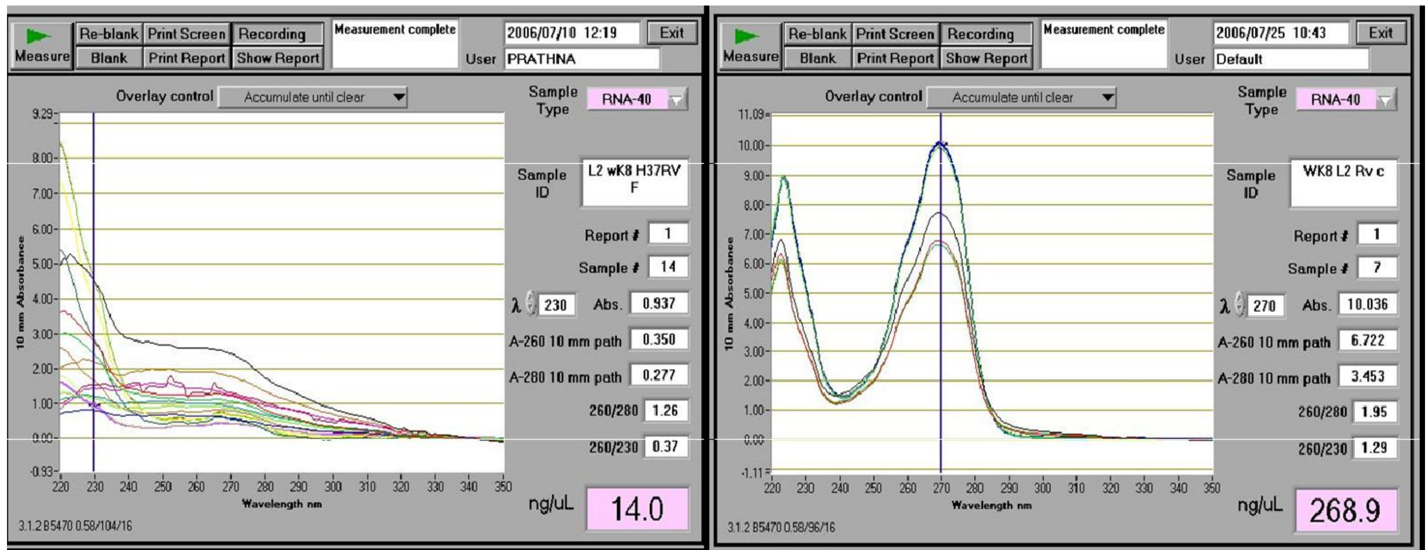


Figure 2. Nanodrop-1000 readings for RNA dissolved at 55°C (A) and the same sample after heating at 60°C (B).

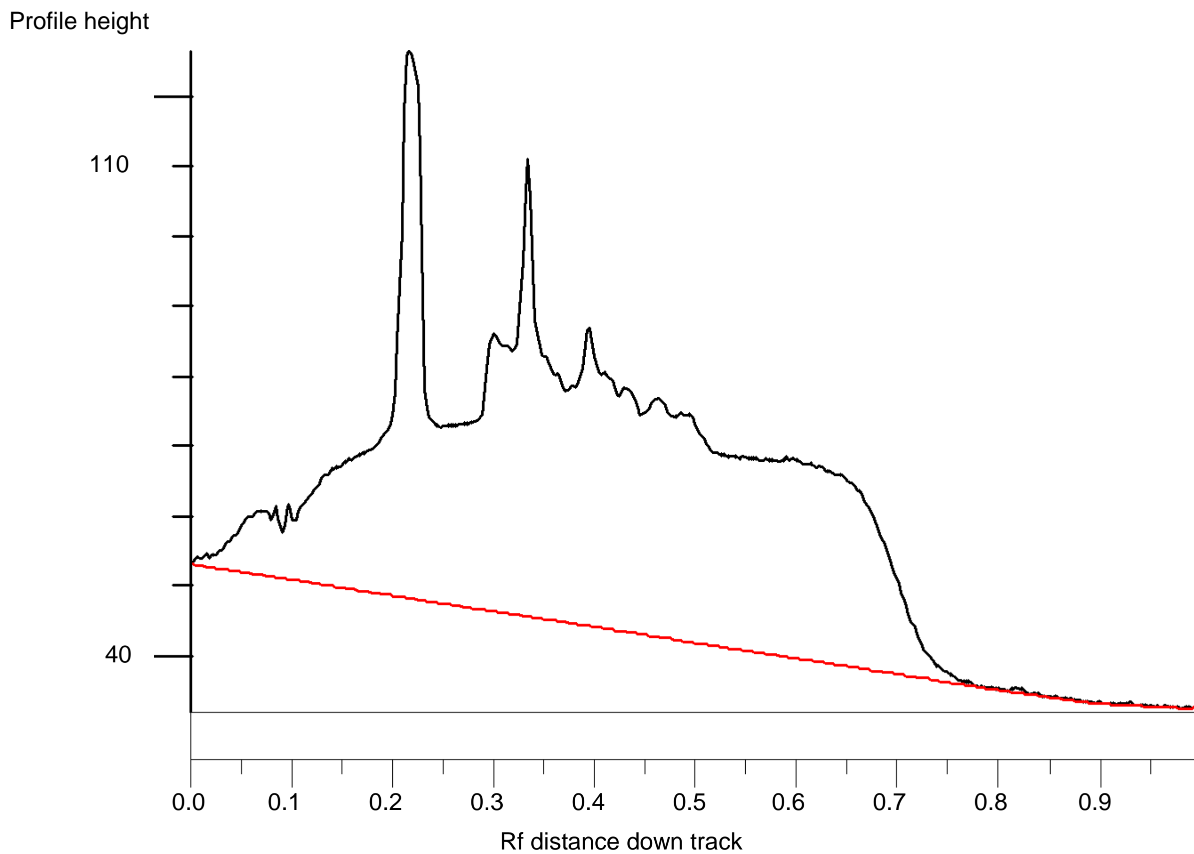
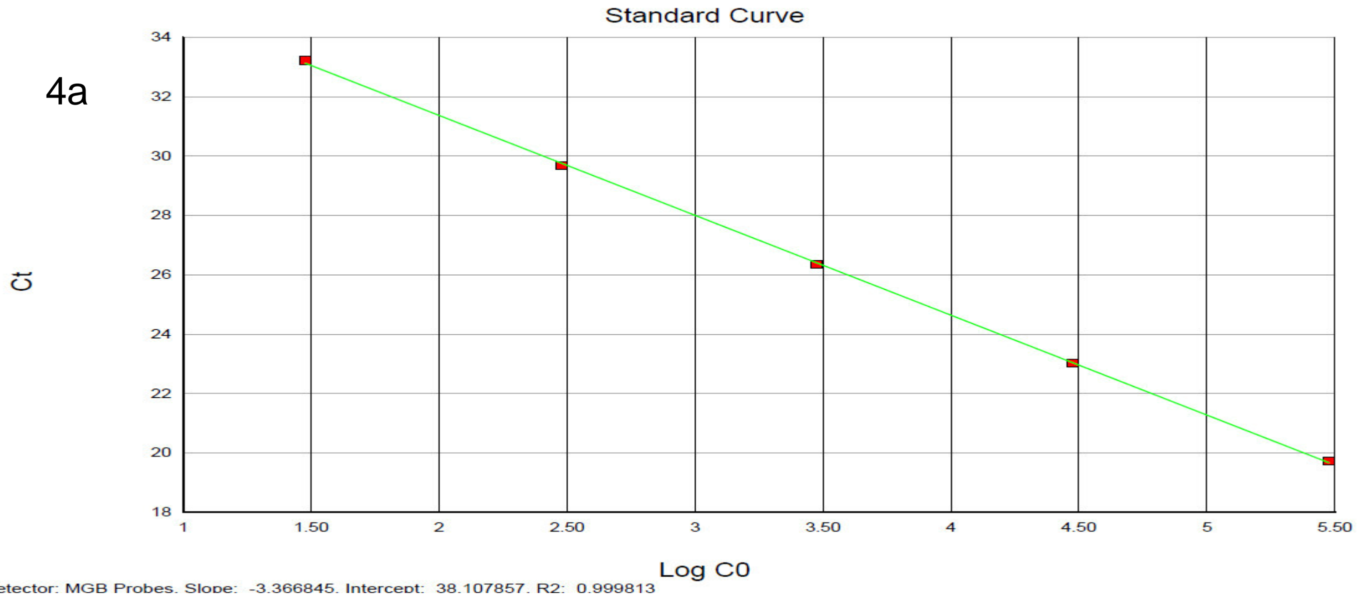
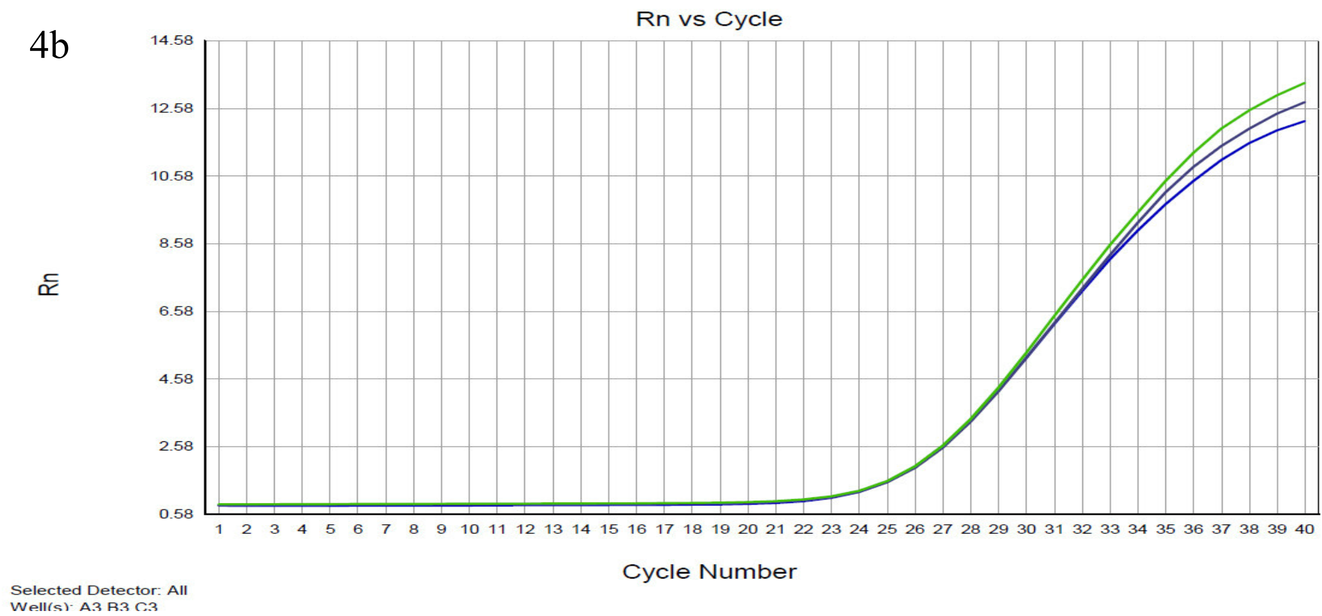


Figure 3. RNA bands present on a 2% agarose gel and Graphical representation of 15 µg optical technology of the Gene Genius viewing system.





Figures 4a and b. Standard curve and Amplification plot for fdxA gene.

A total volume of 50 µl containing 224.7 ng/l of RNA was obtained from 3.1 x 10^8 cells. The purified RNA was stored at -80°C. Using Taqman® (Applied Biosystems) reagents a gene expression ratio of - 2.094 (with a standard error of -0.068), was determined as an average of 9 data points (3 experiments in triplicate). The range showed a minimum of -2.318 and a maximum of -1.809 with 95% confidence intervals from - 2.225 to -1.936. A representative sigmoidal amplification plot and standard curve are given in Figures 4a and 4b respectively. Statistical parameters were determined in InStat V3. Figure 4a shows triplicate fdxA amplification curves with threshold cycles of 25.09, 25.21 and 25.15, respectively.

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