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Isolation of High Quality Total RNA for Transcriptome Study from Thermally Stressed Culture of *Klebsiella pneumoniae* Containing Exogenous RNase Activity

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High quality total RNA isolation is difficult due to exogenous and endogenous RNase activity of *Klebsiella pneumoniae*. Guanidium isothiocynate (GuSCN) is commonly used in RNA purification protocol. Guanidium isothiocynate based method is effective when working with tissues that have high level endogenous RNase. During isolation procedure exogenous RNase degrades the total RNA, therefore, the procedure which can also inhibit exogenous RNase activity is required. Sarkosyl based method is effective for both exogenous and endogenous RNase. Three commercially available kits namely Trizol (GuSCN based), mirVANA RNA isolation kit (GuSCN based) and Ribopure Bacteria Kit (Sarkosyl based) was used for isolation of total RNA. Out of these methods, Ribopure bacteria kit gave high quality of total RNA from thermally stressed (15⁰C and 45⁰C) grown bacterial culture.

Key words: *Klebsiella pneumoniae*, RNA, mRNA, Exogenous RNases, Sarkosyl.

Klebsiella pneumoniae belonging to family enterobacteriaceae is a gram negative, rod shaped, facultative anaerobic and opportunistic pathogenic bacteria. It is found in respiratory, intestinal and urinogenital tracts of animals and human or it may be exogenously acquired within the natural environment[1]. Total RNA isolation from this bacterium is merely a formidable task as exogenous and endogenous RNase activity has been reported[2]. Disruption of the cells, however, inevitably results in rapid mixing of RNA and RNase. RNA degradation can be eliminated if anyhow denature the cellular protein and exogenous and endogenous RNases[3]. Isolation of intact mRNA requires high quality total RNA as it plays a significant role in downstream processing like gene expression profiling, cDNA library construction for SAGE analysis and whole transcriptome analysis on all the next generation sequencing platforms like ROCHE 454, SOLiD analyzer, Illumina genome analyzer etc. In prokaryotes, messenger RNA isolation is also an abstruse process because they have unstable half-lives ranging from 1.5 to 5 minutes[4].

Pure cultures of *Klebsiella pneumoniae* were initially grown at its optimum temperature (35°C). One of them was subjected to a thermal shock at 15°C lower than the optimum temperature and another was subjected at 45°C higher than the optimum temperature. Three different methods namely mirVANA RNA isolation kit (Ambion), Trizol (Invitrogen) reagent based method and Ribopure-bacteria kit were tried out for RNA

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isolation from both the cultures. mRNA isolation was carried out using MICROBExpress bacterial mRNA Enrichment kit (Ambion). The quality and quantity of RNA and mRNA was analyzed by using absorbance ratio (A260/A280 and A260/A230) on Nanodrop 8000 spectrophotometer and RNA integrity number (RIN) on Agilent Bioanalyzer 2100 using the RNA 6000 Pico Lab chip (Agilent Technologies, Santa clara, Palo Alto, CA, USA). The objective of the present study was to isolated of high quality of total RNA by inhibiting the surface and endogenous RNase activity suitable for mRNA isolation.

MATERIALS AND METHODS

Identification and propagation of culture

Pure culture of *Klebsiella pneumoniae* was isolated from the soil and was confirmed by gram staining. Gram negative, rod shaped cells of *Klebsiella pneumoniae* were visualized by oil emulsion microscopy on light microscope (Fig 1). Further confirmation of the culture was also done by microbial identification using 16S rDNA sequencing on 3730xl DNA analyzer at our genomic center. The culture was first streaked on nutrient agar plate by quadrant streaking method and incubated at 35ºC for 48 hours, which is its optimum growth temperature [5]. Single colony was taken from the agar plate and inoculated in 5 ml of tryptic soya broth in a borosil tube for 5-6 hours at 35ºC to obtain actively growing cells as seed culture. From which 300µl of inoculum was inoculated in 5ml of tryptic soya broth containing borosil tube in duplicate and incubated at 35ºC for 4 hours. Then one tube was subjected to 15ºC temperature in a refrigerated shaker and another tube was subjected at 45ºC in water bath for 15 minutes. 1ml of grown culture was taken in 1.5ml of RNase free tube and centrifuged at 5000 rpm for 5 minutes for harvesting. The pelleted cells were then proceeded for total RNA isolation.

Trizol method

1ml of Trizol reagent was added to the bacterial pellet (approximately 10^6 cells), vortexed and incubated at room temperature for 5 minutes. Then 200µl of chloroform was added and vortexed for 30 seconds, and incubated at room temperature for 5 minutes followed by centrifugation at 12000 rpm for 10 minutes at 4°C for phase separation. The aqueous phase was then transferred in 1.5ml RNase free tube followed by the addition of 0.5 volume of isopropyl alcohol. The tube was mixed gently and incubated at -20°C for 30 minutes. The tube was centrifuged at 14000 rpm for 15 minutes at 4°C to pellet out the total RNA. Supernatant was discarded and the pellet was washed with 1ml of 75% ethanol and centrifuged at 10000 rpm for 5 minutes at 4°C. Supernatant was discarded, the pellet was air dried, dissolved in 30-50µl of RNase free water and incubated it at 55°C for 10 minutes.

mirVANA RNA isolation kit (Ambion)

Bacterial cell pellets (approximately 10^6 cells) were dissolved in 600 µl lysis buffer/binding solution by vortexing to completely lyse the cells and to obtain homogeneous lysate. Total RNA was isolated as per manufacturer's protocol.

Ribopure-bacteria kit for RNA isolation

250µl of zirconia beads were taken into 0.5ml screw cap tube for each sample. 350µl of RNAWIZ was added to the pelleted cells and resuspended by vigorous vortexing for 10-15 seconds. These resuspended cells were transferred to the zirconia beads followed by beating for 10 minutes on vortexer with vortex adaptor. Then the beads were pelleted by centrifugation at 14000 rpm for 5 minutes at 4ºC and lysate was transferred to a new tube. 0.2 volumes of chloroform was added, mixed well and incubated for 10 minutes at room temperature followed by centrifugation at 14000 rpm for 5 minutes at 4ºC for phase separation. Aqueous phase was transferred to a new RNase free tube and 0.5 volume of absolute ethanol was added followed by incubation at room temperature for 10 minutes for precipitation. Lysate/ethanol mixture was loaded on the filter cartridge and centrifuged at 14000 rpm for 2 minutes, flow through was discarded. The filter containing total RNA was washed with 700µl of wash solution-1 followed by washing twice with 500µl of wash solution 2/3. Then the filter was spun at 14000 rpm for 2 minutes for removing any residual wash solution. Total RNA was eluted twice with warm 25µl of elution solution in new RNase free collection tube.

Quantification and qualification of RNA

Determination of quantity of isolated total RNA was done by Nanodrop 8000 spectrophotometer, A260/280 ratio and RNA concentration was tabulated in results (Table1) and quality was analyzed on 1% denaturing agarose

gel, further RNA integrity number (RIN) was analyzed on Agilent Bioanalyzer 2100.

Messenger RNA Isolation

By using readily available kits in market, mRNA can be isolated easily but the quality of mRNA is of most important. MICROBExpress bacterial mRNA Enrichment kit (Ambion) were used for mRNA isolation according to manufacturer's protocol. 8µg of total RNA was used for mRNA isolation. Quality and quantity was analyzed on Agilent 6000 RNA pico chip of the isolated mRNA of 15°C culture and 45°C culture.

RESULTS AND DISCUSSION

The difficulty in RNA isolation is that most ribonucleases are very stable and active enzymes that require no cofactors to function. The first step in all RNA isolation protocols therefore involves lysing the cell in a chemical environment that results in denaturation of ribonuclease. The RNA is then fractionated from the other cellular macromolecules under conditions that limit or eliminate any residual RNase activity[6]. The cell type from which RNA is to be isolated and the eventual use of that RNA will determine which procedure is appropriate. Isolation of total RNA from *Klebsiella pneumoniae* using different methods were analyzed on the basis of quantity and quality in 1% denature agarose gel, Nanodrop 8000 spectrophotometer and RNA integrity on Agilent Bioanalyzer 2100. Agarose gel profile of total RNA clearly indicated that mirVANA RNA isolation kit (Ambion) and Trizol reagent (Invitrogen) was not suitable for *Klebsiella pneumoniae* (Fig 2). However, mirVANA RNA isolation kit (Ambion) and Trizol reagent (Invitrogen) can isolate high quality of total RNA from other bacterial sp. but in gram negative

Table 1. Yield and quality of total RNA determined on Nanodrop 8000

S. No.	Method	Temperature stress treatment	A260/ 280	Concentration (µg)
1	Trizol method	15º for 30 minutes	0.67	32.12
2	Trizol method	45º for 30 minutes	0.73	26.14
3	Combination of RNaseZap and Trizol method	15º for 30 minutes	1.94	21.61
4	Combination of RNaseZap and Trizol method	45º for 30 minutes	1.88	35.13
5	mirVANA RNA isolation kit(Ambion)	15º for 30 minutes	0.58	14.85
6	mirVANA RNA isolation kit(Ambion)	45º for 30 minutes	0.50	21.25
7	Ribopure-bacteria kit	15º for 30 minutes	1.65	12.25
8	Ribopure-bacteria kit	45º for 30 minutes	1.83	9.68

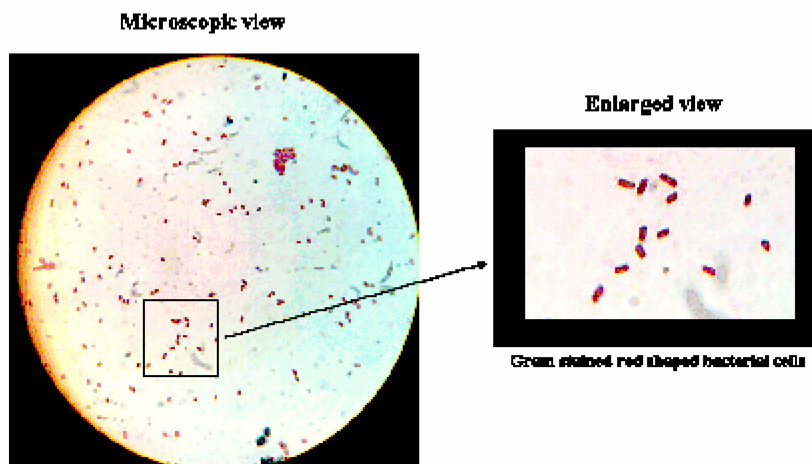
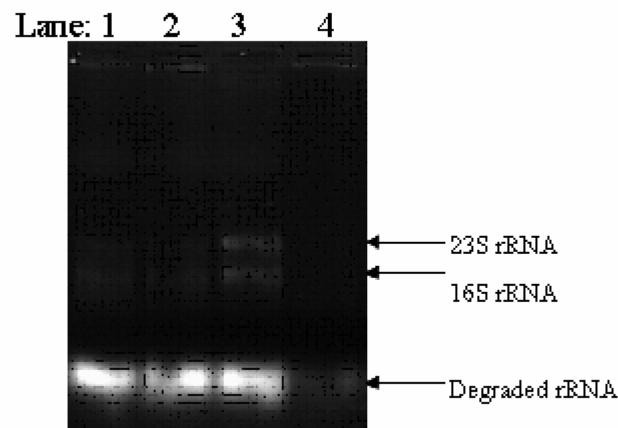


Fig. 1. Gram stained pure culture of *Klebsiella pneumoniae*

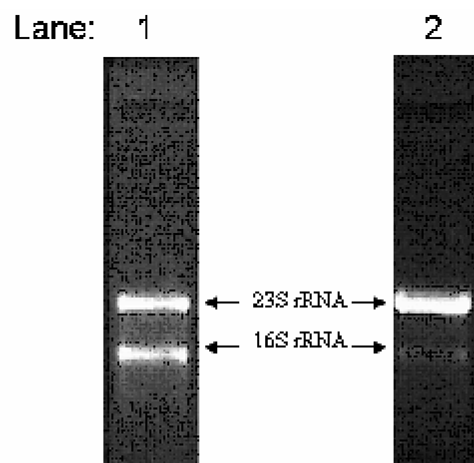
Klebsiella sp., RNase enzyme was found on the cell surface which degraded the RNA during cell lysis[2]. It is necessary to inactivate or remove surface RNase activity to isolate high quality of RNA from such bacteria. Ribopure-bacteria kit (Ambion) which has been specially made for bacterial RNA isolation has given very high quality RNA from 15ºC culture but in case of 45ºC culture, 23S ribosomal RNA band was intact but 16S ribosomal RNA band was faint (Fig. 3). Total RNA isolation was repeated three times for confirming the profile of RNA.

Guanidium isothiocynate (GuSCN) is commonly used in RNA purification protocol, it denatures nucleases rapidly and liberates nucleic acids from bound protein but at lower concentration of GuSCN effect diminishes[7]. Guanidium isothiocynate based method is effective when working with tissues that have high level endogenous RNase[6]. The lysis buffer of mirVANA RNA isolation kit (Ambion) and Trizol reagent (Invitrogen) is also containing GuSCN may not be effectively inactivate RNase on the cell surface



Lane 1: Total RNA isolated by mirVANA kit from 15ºC culture.
Lane 2: Total RNA isolated by mirVANA kit from 45ºC culture.
Lane 3: Total RNA isolated by trizol reagent based method from 15ºC culture.
Lane 4: Total RNA isolated by trizol reagent based method from 45ºC culture.

Fig. 2. Total RNA isolated by mirVANA and trizol reagent based method



Lane 1: Total RNA isolation by Ribopure-bacteria kit from 15ºC culture.
Lane 2: Total RNA isolation by Ribopure-bacteria kit from 45ºC culture.

Fig. 3: Total RNA isolated by Ribopure-bacteria kit

(exogenous). RNAWIZ lysis buffer of Ribopure-bacteria kit (Ambion) is mixture of Phenol, Sarkosyl and HCl. Sarkosyl detergent is a relatively mild chaotropic agent which disrupts the structure of, and denatures, macromolecules such as proteins and nucleic acids. Chaotropic solutes increase the entropy of the system by interfering with intramolecular interactions mediated by non-covalent forces such as hydrogen bonds, van der Waals forces, and hydrophobic effects. Macromolecular structure and function is dependent on the net effect of these forces, therefore it follows that an increase in chaotropic solutes in a biological system will denature macromolecules, reduce enzymatic activity and induce stress on a cell[8]. Thus, Sarkosyl detergent inactivated both exogenous and endogenous RNase activity in the bacterial culture of *Klebsiella pneumoniae*.

The quality of RNA was further confirmed by RNA integrity number (RIN) on Agilent 2100 Bioanalyzer. The RNA integrity number (RIN) is an algorithm for assigning integrity values to RNA measurements. The RIN algorithm is based on a selection of features that contribute information about the RNA integrity. RIN value is always calculated on the scale of 10, RNA with RIN value 10 is considered to be the best and RIN 1 is the worst. The height of the 23S/28S peak contributes additional information about the state of the

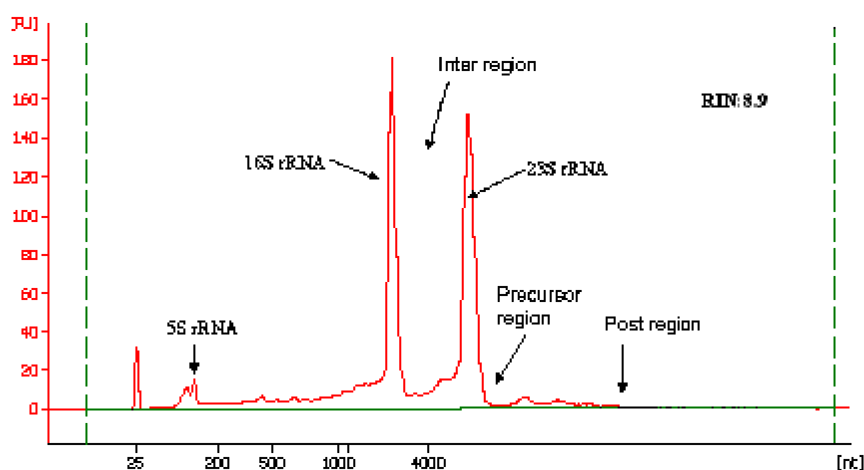


Fig. 4. Bioanalyzer profile of Total RNA isolated by Ribopure-bacteria kit from 15ºC culture

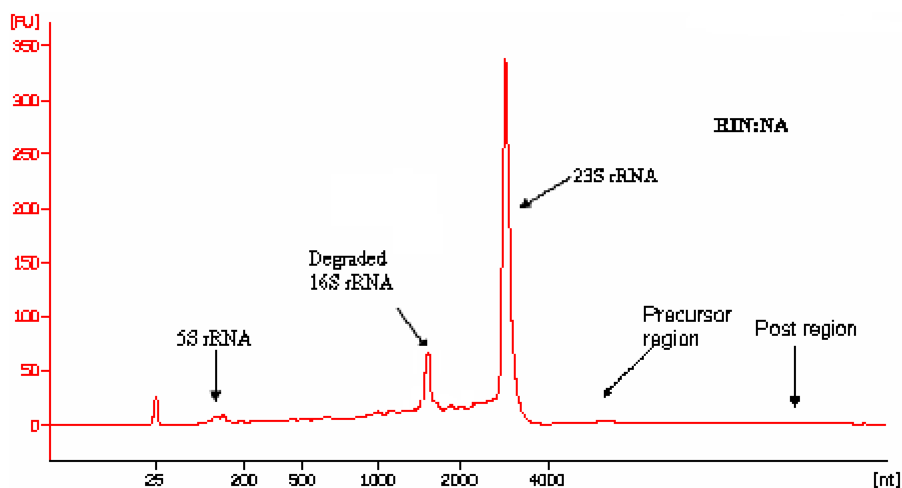


Fig. 5. Bioanalyzer profile of Total RNA isolated by Ribopure-bacteria kit from 45ºC culture

degradation process, i.e. during degradation, the 23S/28S band disappears faster than the 16S/18S band. Therefore, it allows detection of a beginning degradation[9]. Bioanalyzer profiles of the total RNA isolated by Ribopure-bacteria kit is showing distinct peaks of 23S and 16S rRNA in 15ºC culture with RIN value 8.9 (Fig 4). In case of 45ºC culture distinct peak of 23S rRNA but 16S rRNA peak height is very low with RIN is NA (not applicable) (Fig 5). The faint band/low peak height of 16S rRNA due to thermal injury resulted in degradation of the 30S subunit whereas 23S rRNA is retained after 15 min of heating at 45ºC [10, 11]. This method had given good quality total RNA which was suitable for mRNA isolation.

Generally mRNA from any cells, either eukaryotes or prokaryotes, is highly unstable. In *Klebsiella pneumoniae,* half life of mRNA is ranging from 1.5 to 5 minutes [4]. Several kits for the mRNA isolation are available in the market for

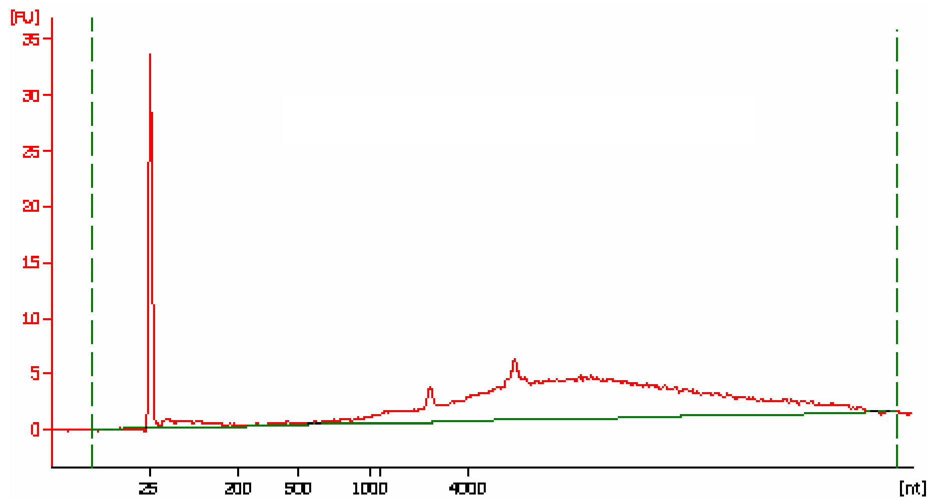


Fig. 6. Bioanalyzer profile of mRNA isolated by MICROBExpress bacterial mRNA enrichment kit from 15ºC culture

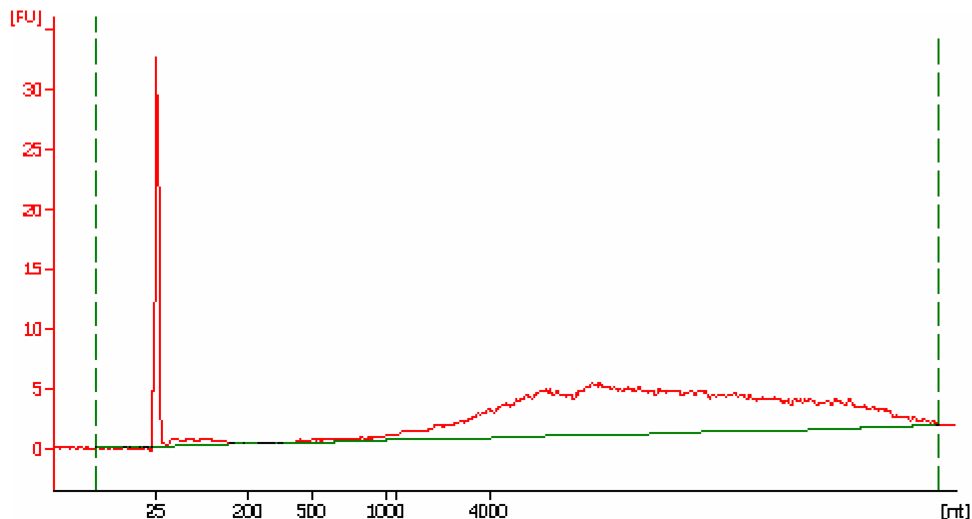


Fig. 7. Bioanalyzer profile of mRNA isolated by MICROBExpress bacterial mRNA enrichment kit from 45ºC culture

prokaryotes namely RiboMinus Bacteria Module (Invitrogen), MICROBExpress bacterial mRNA Enrichment kit (Ambion), which remove ribosomal RNA from total RNA using ribosomal RNA specific probes. MICROBExpress bacterial mRNA Enrichment kit (Ambion) was used for mRNA isolation which given in the range of 0.7-1 µg mRNA from 8 µg of total RNA. Quality of isolated mRNA was analyzed on Agilent 2100 Bioanalyzer using Agilent 6000 pico chip (Fig. 6 and Fig. 7). The bioanalyzer profile clearly indicated enrichment of mRNA and removal of 23S and 16S rRNA. This mRNA can be used in further downstream application like Transcriptome sequencing using next generation platforms, SAGE and differential gene expression study etc. Sarkosyl based lysis method in Ribopure-bacteria kit is able to inactivate both exogenously and endogenous RNase proved the best for RNA isolation from both the thermal stressed cultures of *Klebsiella pneumoniae*.

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