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COMPARISON OF RNA EXTRACTION METHODS FOR TRANSCRIPT ANALYSIS FROM THE PSYCHROPHILIC YEAST, *Glaciozyma antarctica*

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ABSTRACT

To survive in extremely cold environments, psychrophilic microorganisms produce exopolysaccharides (EPS), which are carbohydrate polymers that constitute a substantial component of the extracellular polymers surrounding cells of these microorganisms. EPS can interfere with RNA extraction and decrease the purity of the RNA extracted from EPS producing microorganisms. In this work, six commercial RNA extraction kits and two published protocols for RNA extraction were evaluated for total RNA extraction from the psychrophilic yeast, *Glaciozyma antarctica*. All the protocols were optimised to obtain the highest quality of total RNA. The results show that all of the tested commercial kits and the tested conventional methods yielded RNA from *G. antarctica*, albeit with varying quality. The protocol that utilises TRIzol® reagent was the most effective method for isolating total RNA from *G. antarctica* of which this protocol resulted in the highest RNA yield and purity compared to other methods. This method of RNA extraction produced RNA of sufficient quality for reverse transcriptase PCR (RT-PCR) to detect the expression of the *G. antarctica* delta 9-fatty acid desaturase gene as well as for the construction of a *G. antarctica* cDNA library.

Key words: *Glaciozyma antarctica*, RNA extraction, cDNA library, psychrophiles

INTRODUCTION

Glaciozyma antarctica is an obligate psychrophilic yeast that grows in extremely cold environments (Hashim *et al*., 2013). *G. antarctica* lives in the Antarctic marine waters where temperatures range from -2.2°C to 4°C. There are two main challenges for psychrophilic organisms that must survive in cold environments, the first is that low temperatures decrease the rates of biochemical reactions and thereby affect cell growth; the second challenge involves the viscosity of these aqueous environments (D'Amico *et al.*, 2006). To thrive in extremely cold environments, psychrophiles have evolved a complex range of adaptation strategies, which include the following: secreting antifreeze proteins (Kawahara *et al.*, 2007) and exopolysaccharides (EPS) (Krembs *et al.*, 2002), maintaining membrane fluidity by expressing fatty acid desaturase enzymes (Los and Murata, 2004), producing cold-shock and heat-shock proteins that function as chaperones and producing cold-adapted enzymes (D'Amico *et al.*, 2006). To understand how *G. antarctica* adapts to cold, harsh environments, one must understand every aspect of its physiology and biology.

One way to understand biological changes that occur in response to the surrounding environment involves examining gene expression. Generally, transcriptomics profiling is useful for providing insights into biological pathways and molecular mechanisms that change in response to environmental changes. However, to perform gene expression analysis, one needs to develop or utilize a reliable, rapid and efficient method for RNA isolation. Obtaining high quality, intact RNA is the first and often the most critical step in performing both the steps for gene expression experiments and

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the experiments themselves, including transcript sequencing via second generation sequencing platforms, DNA micro-array analysis, reverse transcriptase-polymerase chain reaction (RT-PCR), Northern blot analysis and cDNA library construction (Fleige and Pfaffl, 2006). Assessing RNA integrity is a critical first step in obtaining meaningful gene expression data. Working with low-quality RNA may strongly compromise the experimental results of downstream applications, which are often labour intensive, time consuming and very expensive (Imbeaud *et al.*, 2005). In addition, degraded RNA or RNA that contains impurities often performs poorly in most enzymatic applications.

A number of protocols have been described as suitable for yeast or fungal RNA isolation (Sokolovsky *et al.*, 1990; Mukhtar *et al.*, 1998; Bolano *et al.*, 2001; Kamaruddin *et al.*, 2007), and many of these protocols have been applied to commercial kits, which are supplied by various companies. However, many protocols, while apparently suitable for fungi, may not be versatile or efficient for extracting RNA from certain types of yeast, especially psychrophiles. RNA isolation in *G. antarctica* is complicated by the abundance of exopolysaccharides in the cell wall, which can interfere with the extraction process and affect the quality of the extracted total RNA. In addition, slow growing *G. antarctica* cells contain modest concentrations of mRNA, and several extraction steps are required to purify sufficient amounts of RNA for the construction of cDNA libraries (Junttila *et al.*, 2009). Moreover, these protocols can be tedious and time consuming. Hence, in this paper, we report on the performance of several RNA extraction protocols for isolating RNA from *G. antarctica* using different systems.

MATERIALS AND METHODS

Yeast cultures

The *G. antarctica* sample used was a kind gifted by Prof. Dr. Nazalan Najimudin from the University of Sains Malaysia, Penang, Malaysia (Hashim *et al.*, 2013). This yeast sample was isolated from sea ice collected at the Casey Research Station, Antarctica. Cells were cultivated on yeast peptone dextrose agar (YPD agar: 0.5% yeast extract, 1% peptone, 2% glucose and 1% bacteriological agar) at 4ºC. Cells grown for active usage were plated once every two weeks. Stock cultures were maintained at -80°C in 20% glycerol.

Growth conditions and sample preparation

A total of 1 X 10^6 cells/ml were transferred from a 3 ml starter culture into 50 ml YPD broth and grown at 4ºC and 180 rpm for 6 days until the culture reached the mid-log phase. Cells were collected by centrifugation for 5 min at 3,000 x g. The pellet was mixed with the remaining supernatant, and the sample was immediately frozen in liquid nitrogen or stored at -80ºC until needed. Approximately 0.5 g of the frozen samples was powdered in a dismembrator (B. Braun Biotech International, Germany) in the presence of liquid nitrogen to disrupt the cells. All experiments were performed in triplicate.

Isolation of RNA using the Ribopure™ Yeast kit (Ambion, Applied Biosystems, USA)

The Ribopure™ Yeast kit is a rapid RNA isolation kit that combines the disruption of yeast cells with Zirconia Beads, phenol extraction of the lysate, and a glass-fibre filter to purify the RNA. Up to 3 X 10^8 cells can be processed at a time. A total of 480 µl of lysis buffer (containing guanidine thiocyanate), 48 µl of 10% SDS and 480 µl of phenol:chloroform:isoamylalcohol were added in this order to the powdered cells, which were then resuspend by vortexing vigorously for 15 sec. The mixture containing cells and lysis reagents was transferred to a 1.5 ml screw cap tube containing 750 µl (to a height of 2.5 cm) of cold Zirconia Beads. The cells were agitated by vortexing for 10 min to lyse the cells. This step was followed by centrifugation for 5 min at 16,000 x g at room temperature to separate the aqueous phase (containing the RNA) from the organic phase. The lysate was then centrifuged to separate the aqueous phase, which contains the RNA, from the lower organic phase, which contains proteins, polysaccharides, and other cellular debris. The aqueous lysate was diluted with 1.9 ml Binding Buffer and 1.25 ml 100% ethanol and was then drawn through a glass-fibre filter, which immobilises the RNA via centrifugation. Contaminants were washed from the filter, and the RNA was eluted in a solution of low ionic strength. Residual DNA was removed by treating the RNA with the Ambion DNA-free™ reagent (included in the kit).

Isolation of RNA using the RNeasy Mini Kit (Qiagen, USA)

The RNeasy Mini Kit combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialised high-salt buffer system allows up to 100 µg of RNA to bind to the RNeasy silica membrane. This protocol is for the purification of total RNA in up to 5 x 10^7 yeast cells. Lysed frozen cells were combined with 600 µl Buffer RLT (with 1% β-mercaptoethanol added; guanidine-thiocyanate-containing buffer) and vortexed to resuspend the cell pellet. The lysate was transferred to a new

microcentrifuge tube and centrifuged for 2 min at full speed. The supernatant was transferred to a new tube, and 1 volume of 70% ethanol was added. The sample was mixed by pipetting, transferred to an RNeasy spin column and placed in a 2 ml collection tube. This tube was centrifuged for 1 min at 8,000 X g. The flow-through was discarded. This process was repeated for the rest of the sample volume. A total of 700 µl of Buffer RW1 was added to the spin column and centrifuged for 1 min at 8,000 x g to wash the spin column, followed by a final wash with 500 µl Buffer RPE (ethanol added). The spin column was placed in a new 1.5 ml collection tube and 30-50 µl RNase-free water was added to elute the RNA. DNase digestion was performed before the RNA clean-up step, which is used to purify RNA from enzymatic reactions (e.g., DNase digestion, RNA labelling) or to desalt RNA samples (Qiagen RNeasy Mini Handbook).

Isolation of RNA using the PureLink™ RNA mini Kit (Invitrogen, USA)

Using this protocol, samples were lysed and then homogenised in the presence of Lysis Buffer, which contains 1% β-mercaptoethanol and guanidium isothiocyanate, a chaotropic salt that is capable of protecting RNA from endogenous RNAses. After homogenisation, 1 volume of 70% ethanol was added to the sample. The sample was then processed through a Spin Cartridge containing a clear silica-based membrane to which RNA binds. Impurities were removed by washing the cartridge with Wash Buffer I and Wash Buffer II. The purified total RNA was then eluted in RNAse-free water and stored at -80ºC. To remove DNA contamination after purification, the RNA was incubated with 10x DNase I buffer and DNase I for 15 min at room temperature. Heat inactivation solution was used to inactivate the DNase I and was followed by an RNA clean-up step. Finally, the RNA was eluted with RNase-free water and the sample was stored at -80ºC.

Isolation of RNA using the TRIzol® Reagent (Invitrogen, USA)

TRIzol® Reagent is a monophasic solution of phenol, guanidine isothiocyanate and other proprietary components that facilitate the isolation of a variety of RNA species from difficult samples. The reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components during sample homogenization. Following cell disruption by a mortar and pestle, the fine powder was incubated with TRIzol® Reagent at room temperature for 5 min to allow for the complete dissociation of nucleoprotein complexes. The homogenates were aliquoted into 1.5 ml tubes and 0.2 ml of chloroform was added for every 1 ml of TRIzol® Reagent. The tubes were shaken vigorously for 15 sec, incubated at room temperature for 2-3 min and then centrifuged at 12,000 X g for 15 min at 4ºC. These steps separate the phases; the mixture is separated into a phenol-chloroform phase, an interphase and an aqueous phase. The RNA, which remains exclusively in the aqueous phase, was transferred into a new eppendorf tube before proceeding to the precipitation step, which involved adding 0.5 ml of cold isopropanol for 10 min. After centrifugation at 12,000 X g for 8 min at 4ºC, the pellet was washed with 1 ml 75% cold ethanol and centrifuged for another 5 min at the same speed and temperature as the previous round. The pellet was then dried and dissolved in 40 µl RNase-free water at 60ºC for 10 min. As with the PureLink™ RNA mini Kit, the RNA was treated with DNAse I to remove DNA contamination and was then treated with another clean-up step.

Isolation of RNA using the Agilent Total RNA Isolation Mini Kit (Agilent Technologies, USA)

The Agilent Total RNA Isolation Mini Kit is a phenol-free, spin-column method for the isolation of total cellular RNA. This method employs a unique prefiltration column that removes cellular contamination. Frozen cells were finely ground and homogenised in Lysis Solution (1% β-mercaptoethanol added) and centrifuged through a mini prefiltration column for 3 min at full speed. This step ensures complete homogenization of the tissue and removes cellular contaminants. An equal volume of 70% ethanol was added to the clarified homogenate and incubated for 5 min at room temperature. The ethanol/lysis mixture was transferred to the mini isolation column and centrifuged for 30 sec at full speed. The flow-through was discarded. The RNA-loaded column was washed with 500 µl Wash Solution (ethanol added) and centrifuged for 30 sec at full speed. The flow-through was discarded and the wash step was repeated. Finally, the mini isolation column was transferred to a new 1.5 ml RNase-free final collection tube, and the RNA was eluted with 10-50 µl of nuclease-free water and treated with DNase I and an RNA clean-up step prior to storage at -80ºC.

Isolation of RNA using the GenCatch™ Total RNA Miniprep Kit (Epoch Biolabs, USA)

This protocol employs the use of a simple silica-membrane spin-column that can isolate total RNA without performing the time-consuming steps of phenol/chloroform extraction and ethanol precipitation. The binding capacity of the total RNA mini column is 100 µg of total RNA. The disrupted sample was homogenised in 350 µl RX Buffer (β-mercaptoethanol added) and was centrifuged for 5 min to spin down insoluble materials. The supernatant was mixed with an equal volume of

70% ethanol and transferred to a Total RNA Mini Column and placed in a Collection Tube. The sample was centrifuged for 1 min and the flow-through was discarded. This process was repeated with the same column for the remaining samples. The lysate was then washed with 0.5 ml WS Buffer, centrifuged for 1 min and washed again with 0.7 ml WF Buffer before centrifuging for 3 min to remove residual ethanol. The column was placed in a 1.5 ml RNase-free Elution Tube and eluted with 30-50 µl RNase-free water. Because some genomic DNA can co-purify with RNA, DNase treatment was carried out and followed by an RNA clean-up step.

Isolation of RNA using phenol and LiCl precipitation (Karim *et al.*, 2007)

Cells were homogenised in 0.75 ml lysis buffer (0.6 M NaCl, 10 mM EDTA, 100 mM Tris-HCl pH 8.0, and 4% SDS). An equal volume of phenol was added to the tube, and the sample was vortexed for 15 min followed by a centrifugation step at 13,000 rpm at 4ºC for 10 min. The aqueous layer was transferred to a new tube containing an equal volume of phenol. After centrifugation for 10 min at 10,000 rpm and 4ºC, the aqueous layer was transferred to a new tube that contained a 0.75 volume of 8 M LiCl, and the sample was stored overnight at 4ºC for future RNA precipitation. The pellet was dissolved in a 0.3 volume of RNase-free water, and both 0.03 ml of 3 M sodium acetate (pH 5.2) and 0.75 ml of 100% ethanol were added to each tube. RNA was precipitated by incubating the sample at –20ºC for 2 hr. After centrifugation, the pellet was washed with 70% ethanol, air dried and dissolved in 20 µl of RNase-free water.

Isolation of RNA using the CTAB/NaCl method (Junttila *et al.*, 2009)

A 0.5 g sample was homogenised and transferred to a tube containing 500 µl of extraction buffer (3% CTAB (W/V), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2 M NaCl, 0.5 g/l spermidine, 3% PVP (V/V), and 4% β-mercaptoethanol (V/V)) and was mixed gently. The mixture was incubated at 65°C for 2 min. An equal volume of chloroform/ isoamyl alcohol (24:1 V/V) was added, and the tube was inverted for 5 min. Next, the tube was centrifuged for 20 min at 12,000 g and 4°C. A 0.25 volume of 10 M LiCl (4°C) was added to the supernatant and the sample was stored at 4°C for 2–3 hr. The tube was centrifuged for 30 min at 12,000 g and 4°C. Next, 500 µl 0.5% SDS was added to resuspend the precipitated sample. An equal volume of chloroform/isoamyl alcohol (24:1, V/V) was then added, and the sample was centrifuged for 10 min at 12,000 g and 4°C. Two volumes of chilled ethanol were added next, and the sample was stored at 20°C for 2 hr. After centrifugation, the pellets were washed 3 times with 70% ethanol. The pellets were air dried and suspended in 100 µl of DEPC-treated water.

Assessment of RNA quantity and quality

The yield and purity of RNA isolated from all kits were assessed on a 1.0% agarose gel and quantified by the absorbance ratios of $A_{260/A280}$ and $A_{260/A230}$ using a NanoDrop 1000 (NanoDrop Technologies, Inc., USA). The extractions and readings were performed in triplicate. Additionally, the RNA integrity for all samples was inspected via automated capillary electrophoresis using the Prokaryotic Total RNA Nano Assay with a 2100 Bioanalyzer, which employed electropherogram analysis and a RIN number (Agilent, USA).

Reverse transcriptase PCR (RT-PCR)

To test the quality of the extracted RNA, RT-PCR was carried out using specific intron junction primers that were designed for the amplification of a 170 bp amplicon (without genomic contamination) of a gene that encodes for *G. antarctica* Δ9-fatty acid desaturase. For the RT-PCR analysis, 200 ng of RNA was reverse-transcribed using the Access® RT-PCR kit (Promega, USA) and the following parameters: 45ºC for 45 min (reverse transcription step); 94ºC for 2 min (reverse transcriptase inactivation); 40 cycles of 94ºC for 30 sec, 56.3ºC for 1 min and 68ºC for 30 sec; and a final extension step at 68ºC for 7 min. The amplified product was then analysed by gel electrophoresis on a 2.0% agarose gel containing 10 mg/ml of ethidium bromide and visualised under UV light.

cDNA library construction

G. antarctica was grown as described previously; however, at day 5, the culture was exposed to -12°C for an additional 2 days. Total RNA was extracted using TRIzol® Reagent as described above. The mRNA was isolated from the total RNA with the PolyATtract® mRNA Isolation Systems kit (Promega). A cDNA library was constructed from the mRNA using the CloneMiner cDNA Library Construction Kit (Invitrogen, USA) according to the manufacturer's protocol with some modifications. Next, 5376 *Escherichia coli* transformants (ElectroMax DH10B) containing cDNA inserts from the library were picked, labelled with a BigDye v3.1 reaction (Applied Biosystems, Foster City, USA), and sequenced using an ABI PRISM 3130xl Genetic Analyzer (a capillary DNA sequencing machine). All clones were sequenced from the 5' end of the insert using the M13 forward primer: 5'-GTAAAACGACGGCCAG-3'.

Sequence analysis

The raw data from the chromatogram files were processed by base-calling and were assessed for quality by Phred software (Phred-Phrap Consed package) (Ewing *et al.*, 1998). The low-quality sequences were trimmed with Q20 (99% accuracy). The vector sequences were screened with the CROSS_MATCH program. Next, all the sequences were clustered, aligned and analysed using the StackPACK v2.2 pipeline web-based interface, which determined unique transcript identities. BLASTX (Altschul *et al.*, 1990) was used to compare the *G. antarctica* sequences to the NCBI non-redundant protein sequence database.

RESULTS AND DISCUSSION

Exopolysaccharides are important extracellular substances that aid in the winter survival of sea ice organisms and should be considered in addition to intracellular adaptations to cold temperatures and the property of high salinity (Krembs *et al.*, 2002). A high concentration of EPS on the surface of *G. antarctica* makes obtaining pure RNA from the RNA extraction process difficult. Polysaccharide contamination is always a problem when extracting fungal or yeast genetic material. Moreover, polysaccharides can interact with nucleic acids by forming insoluble complexes that can affect the yield and quality of RNA (Chirgwin *et al.*, 1979).

With an appreciation for the importance of RNA quantity and quality for many downstream processes, the best method for extracting total RNA from *G. antarctica* was examined. Eight methods of RNA extraction: six commercial kits from Ambion (Ribopure™ Yeast kit), Qiagen (RNeasy Mini Kit), Invitrogen (TRIzol® reagent and PureLink™ RNA mini Kit), Agilent (RNA Isolation Mini Kit), and Epoch Biolabs (GenCatch™ Total RNA Miniprep Kit), and two conventional methods including LiCl precipitation and the CTAB/NaCl method were examined. All protocols, with the exception of the Ribopure™ Yeast kit, were modified by adding a DNAse treatment step and further purification with the Qiagen RNeasy clean-up kit. These additional steps produced two distinct bands with no genomic contamination and small RNAs; however, a marked decrease in RNA yield was observed following these modifications (Fig. 1). The RNA that was extracted using the Ribopure protocol was only subjected to a DNAse treatment, which was included in the kit. Table 1 summarises the differences between the eight protocols.

Each RNA isolation system employs specific mechanisms to extract pure RNA. Most of the protocols employ a chaotropic agent, such as guanidine thiocyanate or β-mercaptoethanol, which can denature proteins and RNAses in addition to isolating RNA (Chomczynski and Mackey, 1995). One of the unique features of the Ribopure™ yeast kit is the use of zirconia beads to disrupt cells.

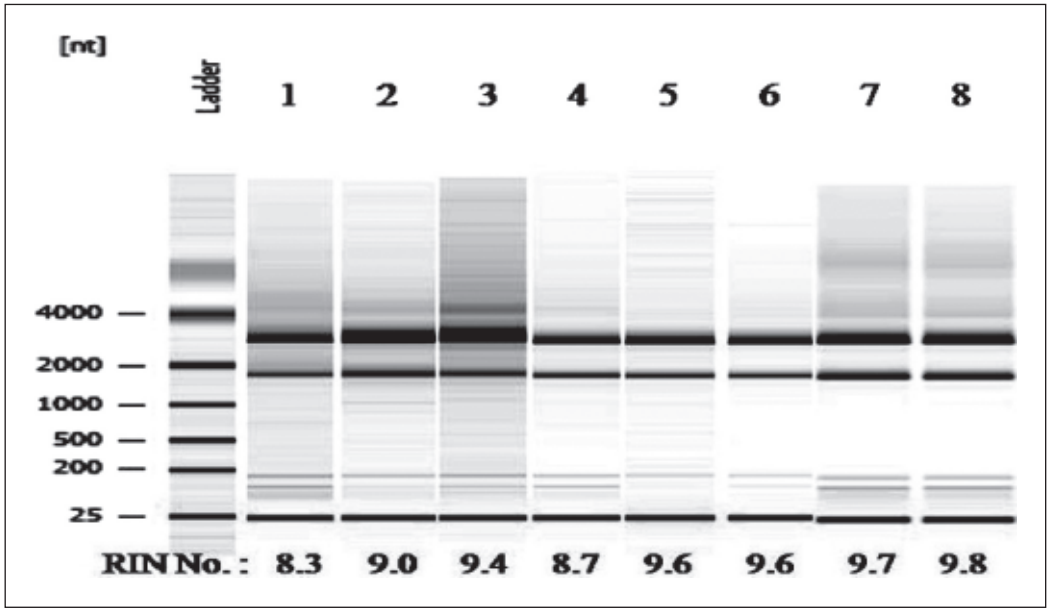

Fig. 1. Detection of total RNA extracted by eight different methods using a Bioanalyzer RNA Nano chip. Lane 1: RNA extracted with the Ribopure™ Yeast kit; Lane 2: RNA extracted with TRIzol® Reagent; Lane 3: RNA extracted with the GenCatch™ Total RNA Miniprep Kit; Lane 4: RNA extracted with the Agilent Total RNA Isolation Mini Kit; Lane 5: RNA extracted with the PureLink Mini Kit (Invitrogen); Lane 6: RNA extracted with the RNeasy Mini Kit (Qiagen), Lane 7: RNA extracted by phenol/LiCl precipitation (Karim *et al.*, 2007); and Lane 8: RNA extracted by the CTAB/NaCl method (Junttila *et al.*, 2009). The RIN number for each RNA preparation is below each lane.

Table 1: Summary of the different RNA isolation procedures

Methods	RNA extraction technology	Average yield (µg/mL)	$A_{260/280}$	$A_{260/230}$	Estimated extraction time from frozen cells (hours)
Ribopure™ Yeast Kit#	Zirconia beads, phenol extraction, glass-fibre filter	2.80 ± 0.10	2.20 ± 0.02	2.15 ± 0.01	1 – 2
PureLink™ RNA Mini Kit	Column based system	1.80 ± 0.30	1.90 ± 0.01	2.08 ± 0.02	1 – 2
TRIzol® reagent	Phenol based	3.30 ± 0.07	2.11 ± 0.03	2.18 ± 0.20	2
RNeasy Mini Kit	Silica-membrane spin-column	0.70 ± 0.10	2.22 ± 0.01	2.38 ± 0.13	1 – 2
Agilent Total RNA Isolation Mini Kit	Spin-column, phenol-free	0.30 ± 0.07	2.14 ± 0.02	1.88 ± 0.05	1 – 2
GenCatch™ Total RNA Miniprep Kit	Silica-membrane, spin-column	0.43 ± 0.10	2.20 ± 0.02	2.19 ± 0.13	1 – 2
Phenol/LiCl Precipitation	Phenol based	0.70 ± 0.20	2.10 ± 0.80	2.11 ± 0.50	48
CTAB/NaCl Method	Phenol based	0.80 ± 0.40	2.14 ± 0.30	2.20 ± 0.40	6 – 7

#Ribopure™ – Yeast kit included the DNase I treatment Reagents

Zirconia beads are 50% more dense than glass beads, which is ideal for difficult tissues. Most other kits used in this work (except TRIzol® reagent) exploit a column-based membrane, which is reported to be selective towards RNA, to purify RNA. However, one drawback of a column-based system is the possibility of overloading the column, which can cause the column to clog or can prevent the RNA from binding efficiently, thus lowering the yield of RNA. Therefore, using the appropriate amount of cells to maximize the column binding capacity is critical. All kits except the Agilent Total RNA Isolation Mini Kit employ the use of a chemical reagent such as phenol or guanidine thiocyanate, which results in a higher recovery of RNA with high purity compared to a column-based system.

Table 1 shows the RNA concentration as well as the results of the equality assessment using the NanoDrop 1000. Using TRIzol® Reagent resulted in the highest average yield of RNA (3.30 ± 0.07 µg/ml), followed by the Ribopure™ yeast kit (2.80 ± 0.10 µg/ ml of total RNA). Total RNA for most samples showed high purity but low protein and polysaccharide contamination, which were established by the $A_{260/280}$ and $A_{260/230}$ ratios. Optical density ratios at $A_{260/280}$ and $A_{260/230}$ nm for all the samples were well within the acceptable ranges of 1.60-1.90 and >2.0, respectively (Sambrook *et al.*, 1989). Hence, we conclude that TRIzol® Reagent gave the highest yield with relatively good quality followed by the Ribopure™ Yeast kit, which had a slightly lower yield than using TRIzol® Reagent but had higher quality.

The RNA samples were then inspected visually by electrophoresis on a 1% agarose gel and were subjected to automated capillary electrophoresis using the Total RNA Nano Assay with the 2100 Bioanalyzer to verify DNA genomic contamination and intact RNA. Figure 1 shows the Bioanalyzer profiles of RNA extracted by eight different methods. Virtually all protocols gave two distinct bands, which represented 18S and 28S ribosomal subunits, and no genomic contamination or RNA degradation was observed. The RNA Integrity Number (RIN), which indicates the state of RNA degradation, is also reported for each profile in Figure 1. A RIN of '10' represents perfect RNA with no degradation, whereas a RIN of '1' indicates completely degraded RNA. Furthermore, a high RIN ensures repeatability for future experiments.

The quality of the extracted RNA, RT-PCR was carried out using primers designed for the amplification of a gene that encodes for *G. antarctica* Δ9-fatty acid desaturase. As indicated in Fig. 2, a PCR transcript with a size of 170 bp was detected in the total RNA samples that were isolated from each protocol. The successful amplification of this product suggests that the extracted RNA was essentially free of contaminants that could otherwise inhibit enzymatic reactions and that the RNA was amenable for other RNA profiling or protein expression studies.

In addition, the constructed cDNA library from the total RNA that was extracted using TRIzol® reagent. Started with 12 µg of total RNA to isolate the mRNA, which was of good quality and

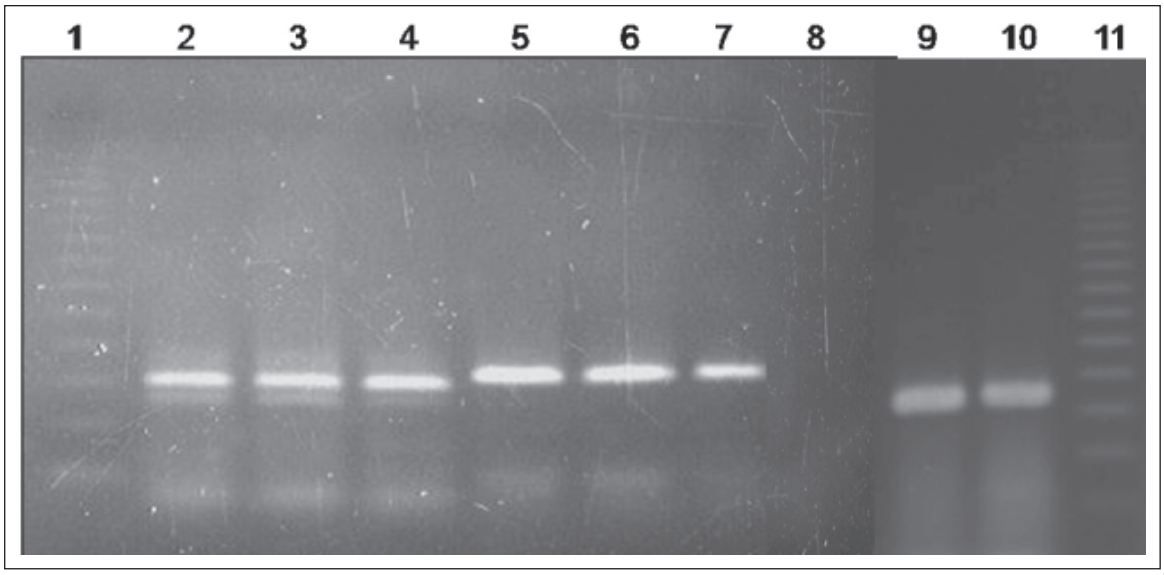

Fig. 2. Gel electrophoresis results of Δ9-fatty acid desaturase RT-PCR using RNA extracted by eight different methods. Lanes 1 & 11: 50 bp marker; Lane 2: amplicon from RNA extracted with the Ribopure™ Yeast kit; Lane 3: amplicon from RNA extracted with the PureLink Mini Kit (Invitrogen); Lane 4: amplicon from RNA extracted with the RNeasy Mini Kit (Qiagen); Lane 5: amplicon from RNA extracted with TRIzol® Reagent; Lane 6: amplicon from RNA extracted with the Agilent Total RNA Isolation Mini Kit; Lane 7: amplicon from RNA extracted with the GenCatch™ Total RNA Miniprep Kit; Lane 8: negative control (no RNA added); Lane 9: amplicon from RNA extracted by phenol/LiCl precipitation (Karim *et al.*, 2007); Lane 10: amplicon from RNA extracted with the CTAB/NaCl method (Junttila *et al.*, 2009).

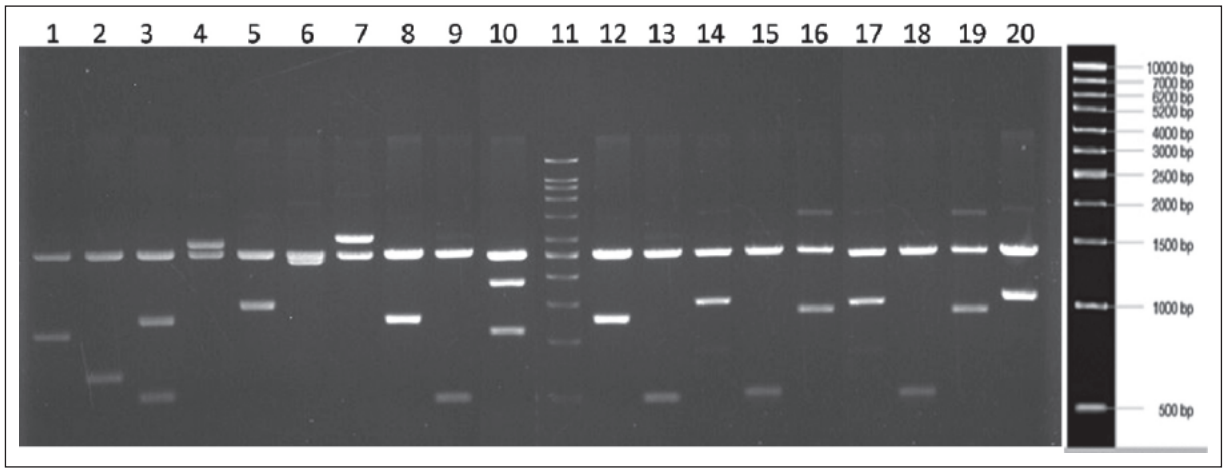

Fig. 3. Gel electrophoresis analysis of a *G. antarctica* cDNA library.
Poly(A)+ RNA was purified from total RNA that was extracted with TRIzol® Reagent and was used for the construction of a cDNA library using the CloneMiner™ cDNA Library Construction Kit (Invitrogen). Plasmid DNA from 16 positive clones was digested with the *BsrG*1 enzyme (New England Biolabs) and electrophoresed on a 1% agarose gel to determine average cDNA insert size. Lanes 1-9 and 12-20: randomly picked cDNA clones; lane 10: vector pDON™ 222 (Invitrogen); lane 11: 1 kb DNA marker (Vivantis, USA). The 2.5 kb band represents the vector backbone.

successfully isolated. Subsequently proceeded with the construction of a cDNA library. The total colony forming units (CFUs) were calculated as 1.61×10^7, which was predicted to be sufficient to represent most of the expressed genes (Sangha *et al.*, 2010) in *G. antarctica*. The range of cDNA insert size was 350 bp to 3000 bp (Fig. 3), and the percentage of recombinant clones was 98%. BLASTX clustering analysis revealed that 95% of the sequences had fungal origin when the arbitrary expectation value of 1^{e-5} was used (data not shown). The sequence data were submitted to the EST database at the National Centre for Biotechnology Information (NCBI).

Identified several genes from the cDNA library that are postulated to be important for *G. antarctica* survival at lower temperatures, such as genes that encode for antifreeze proteins (Accession numbers JZ335201 and JZ339277), Δ9-fatty acid desaturase (JZ338551), Ä12-fatty acid desaturase (JZ335902) and heat-shock proteins (JZ340516, JZ346463,

JZ341754). Antifreeze proteins are important for survival at low temperatures because they inhibit the growth of ice formation around the cell surface (Jia and Davies, 2002). The Δ9 and Δ12-fatty acid desaturase proteins introduce double bonds into the fatty acid chains of membrane lipids, which thereby increase the fluidity of the membrane at lower temperatures (Los and Murata, 2004). These results indicate that total RNA extraction from *G. antarctica* with TRIzol® Reagent retains adequate gene expression for library construction, cDNA sequencing and gene expression analysis.

CONCLUSIONS

In summary, results show that all six commercial kits and the two conventional methods tested can produce quality total RNA. Protocols that use optimised TRIzol® reagent might be superior for the isolation of total RNA from *G. antarctica*; methods using TRIzol® reagent resulted in the highest yield and purity compared to other commercial kits and conventional methods tested. An advantage of this system is the ability to isolate quality RNA from a sample containing a high polysaccharide content, which could then be utilised for RNA profiling techniques. Furthermore, a preliminary EST analysis revealed that total RNA extraction with a TRIzol® reagent method retains several mRNAs that may be involved in the survival mechanisms of *G. antarctica* in cold environments.

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