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Full Length Research Paper

Optimization of yeast (*Saccharomyces cerevisiae*) RNA isolation method for real-time quantitative PCR and microarray analysis

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Quality of the starting RNA is indispensably important for obtaining highly reproducible quantitative polymerase chain reaction (qPCR) and microarray results for all organisms as well as *S. cerevisiae*. Isolating RNA from yeast cells with a maximum quality was especially critical since these cells were rich in polysaccharides and proteins. The method has been optimized through modification pretreatment applications for the isolation of *S. cerevisiae* RNA for qPCR and microarray analysis. Two extraction assay a TRIzol reagent-based method with three pretreatment applications and a commercially available kit with own pretreatment application, were compared for this purpose. Furthermore, the concentration yeast cells and enzyme were controlled in the range of 2×10^6 to 6×10^7 cells and 0.5 to 5 mg/ml, respectively to prevent RNA yields decrease and RNA degradation. Results of RNA isolation of the middle scales of yeast cells and enzyme concentrations which obtained fluxional deduct were not discussed here. Concentration and integrity of RNA samples were determined by µL spectrophotometer and Bioanalyzer, respectively. Quality of cDNA prepared from RNA samples was inspected with amplification using 18SrRNA primers in qPCR reactions. Furthermore, quality of RNA samples were evaluated using quality control parameters associated with performance of the assay and hybridization in microarray experiments. The highest yield and quality of RNA, which was appropriate for reverse transcription, cDNA library construction, qPCR reactions and microarray hybridizations without further processing was obtained by using Protocol C which used highest yeast cell and enzyme concentrations during the pretreatment application.

Key words: RNA isolation, *Saccharomyces cerevisiae*, quantitative PCR, microarray.

INTRODUCTION

Saccharomyces cerevisiae, the baker's yeast, is the first eukaryotic organism whose genome was fully sequenced in 1996 (Goffeau et al., 1996). It has become a favorable model for various studies with its simple genome and short growth period. Considerable experimental tractability of yeast genetics and molecular biology, combined with the detailed acquirements of the yeast genome, has given a remarkable role to *S. cerevisiae* at the center of research ranging from studies of human disease genes to experimental evolution and systems biology (Landry et al., 2006).

Quantitative PCR and microarray assays are now widely used to quantify RNA levels for gene expression studies (Bilgin et al., 2009). Quality of RNA, typically with a ratio of optical densities (OD) at 260 to 280 nm ($A_{260/280}$) between 1.9 to 2.3, is critical for obtaining highly reproducible qPCR and microarray results for all organisms as well as *S. cerevisiae*. Moreover, determining the integrity

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Abbreviations: PCR, Polymerase chain reaction; **cDNA,** complementary **DNA; qPCR,** quantitative **PCR.**

Table 1. The pretreatments used in protocols A, B, C and D.

Protocol	Yeast cell number (ca mg DCW [a])	Lyticase (mg/ml)	TRIzol amount (mL)	Kit employed
A	2×10^6 (1.5×10^{-3})	–	1	–
B	2×10^6 (1.5×10^{-3})	0.5	1	–
C	6×10^7 (4.5×10^{-2})	5.0	1	–
D	2×10^6 (1.5×10^{-3})	0.5	–	High Pure RNA Isolation Kit

(a) DCW: Dry cell weight.

of RNA is a crucial step. Slab gel analysis of total RNA samples using ribosomal ratios often results in an inaccurate assessment of RNA integrity. Bioanalyzer 2100 (Agilent) microfluidic electrophoresis provides a better assessment of RNA integrity by showing a detailed picture of the size distribution of RNA fragments (Braly et al., 2003).

Different methods and commercial kits for RNA extraction require special steps to "break open" the cells. Some of these methods employ hot acid phenol, physical shear forces or enzymatic lysis to break very resilient yeast cell walls (Kohrer and Domdey, 1991; Schmitt et al., 1990; Rivas et al., 2001; Epstein and Butlow, 2003). Once contents of the cells are released, RNA is purified from other cell components with maximum quality and yield. Isolating RNA from yeast cells with a maximum quality is especially critical since these cells are rich in polysaccharides and proteins.

The optimization procedures through modification pretreatment applications reported here allowed for the isolation of *S. cerevisiae* RNA suitable for qPCR and microarray analysis. Two extraction assay a TRIzol reagent-based method with three pretreatment applications and a commercially available kit with own pretreatment application, were compared for this purpose. As a result, we have determined one of the pretreatment applications which used highest concentrations of yeast cells and Lyticase as the most efficient in producing high quality yeast total RNA.

MATERIALS AND METHODS

Strain and growth conditions

The yeast used in this study was commercial baker's yeast (*S. cerevisiae*) obtained from Pakmaya (Turkey) as pressed yeast and was stored at 4°C until used. Yeast cells were grown in YPD broth medium (1% yeast extract, 2% peptone, 2% glucose, pH 5.3) at 30°C with 130 rpm shaking until OD at 600 nm reaches 0.5 (~ 1.5×10^7 cell/ml) which is the recommended mid-log phase for gene expression studies. To obtain initial cultures, an aliquot of 1 ml was transferred to 40 ml YPD medium and cells were grown continuously at 30°C with 130 rpm shaking for 20 h. Grown cells were collected by centrifugation at 3000 ×g for 10 min at room temperature. Each cell pellet was stored at -80°C until RNA extraction.

RNA isolation

Each cell pellet was centrifuged at 2000 ×g for 5 min in a standard table top centrifuge (SIGMA 3K30). The pellet was resuspended in 200 µl PBS buffer (Roche) pH 7.4. Yeast cell concentrations were measured with an AlphaSpect µL Spectrophotometer (AlphaInnotech Inc., USA) at 600 nm. The concentration of yeast cells and enzyme were controlled in the range of 2×10^6 to 6×10^7 and 0.5 to 5 mg/ml, respectively. Two different concentrations (0.5 or 5 mg/ml) of Lyticase (Sigma) were chosen to lyse yeast cells at two different concentrations (2×10^6 or 6×10^7). On the other hand, TRIzol reagent (Invitrogen) was utilized to extract RNA. The pretreatments protocols which were discussed in this report are listed in Table 1.

2×10^6 cells without enzymatic lysis were used for Protocol A and 2×10^6 cells treated with 0.5 mg/ml Lyticase were used for Protocol B. Protocol C involved treatment of 6×10^7 cells with 5 mg/ml Lyticase. All protocols started with addition of 1 ml TRIzol reagent. In all three protocols RNA extraction was done according to method of Chomczynski and Mackey (1987). In protocol D, total RNA was prepared using High Pure RNA Isolation Kit (Roche GmbH, Mannheim, Germany) according to the manufacturer's instructions.

RNA concentrations and the ratio of OD at 260 to 280 nm ($A_{260/280}$) were measured by AlphaSpect µL Spectrophotometer (AlphaInnotech Inc., USA). Agilent 2100 Bioanalyzer and associated RNA 6000 Nano LabChip kit were used to determine the integrity of RNA. All protocols were performed as two parallels with four biological replicates. Each RNA sample was stored at -80°C for further analysis.

cDNA synthesis from total RNA and 18SrRNA gene amplification

cDNA from total RNA was synthesized using Transcriptor High Fidelity cDNA Synthesis kit (Roche GmbH, Mannheim, Germany). LightCycler DNA Master SYBR Green I kit (Roche GmbH, Mannheim, Germany) with 18SrRNA gene specific primers (Iontek Inc., Istanbul, Turkey) were utilized to confirm the quality of RNA obtained from all protocols. All qPCR experiments were conducted in a total volume of 20 µl including 10 ng cDNA. 18SrRNA forward primer 5'–AAACGGCTACCACATCCAAG and 18SrRNA reverse primer 5'–CCCATCCCAAGGTTCAACTA pairs were used for 18SrRNA gene amplification. The optimal amplification efficiency of real-time PCR was obtained at 0.2 µmol forward and reverse primers, 0.2 mM magnesium concentration, 1 × master SYBR Green and 2 µl template in a 20 µl reaction mixture. Cycling conditions for LightCycler® 1.5 thermocycler system is given in Table 2.

cRNA synthesis, biotin labeling and hybridization of Yeast 2.0 GeneChip Array

Complementary RNA (cRNA) synthesis, biotin labeling, hybridization, and scanning were performed according to protocols described in the Affymetrix GeneChip Expression Analysis Technical Manual using Yeast 2.0 GeneChip Array. Hybridization,

Table 2. Cycling conditions for LightCycler® 1.5 thermocycler using SYBR Green I.

Analysis Mode	Cycles	Segment	Target Temperature (°C)	Hold Time	Acquisition Mode
Pre-Incubation					
None	1		95	10 min	None
Amplification					
Quantification	45	Denaturation	95	10 s	None
		Annealing	51	3 s	None
		Extension	72	2 s	Single
Melting Curve					
Melting Curves	1	Denaturation	95	0 s	None
		Annealing	65	15 s	None
		Melting	95	0 s	Continuous
Cooling					
None	1		40	30 s	None

scanning, and preliminary analyses with GeneChip Operating Software 1.4 were performed at Middle East Technical University Molecular Biology and Biotechnology Research and Development Center.

RESULTS

Determination of RNA quality and quantity

In this research, the concentration of yeast cells and enzyme were controlled in the range of 2×10^6 to 6×10^7 cells and 0.5 to 5 mg/ml, respectively to prevent RNA yields decrease and RNA degradation. We have compared different pretreatments, with lowest and highest yeast cells and enzyme concentrations which data were reported here, in combination with an extraction technique using TRIzol reagent and a commercially available kit. RNA isolation results of the middle scales of yeast cells and enzyme concentrations which were obtained fluxional deduct were not discussed here.

High yields of RNA from *S. cerevisiae* cultures, free of DNA, polysaccharide or other contaminants were obtained using Protocol C. Table 3 summarizes the concentrations and recordings of $A_{260/280}$ of total RNA isolated using all protocols in this study. The lowest yield and quality of RNA samples were obtained using Protocol A. Although, RNA yields from Protocol B and C look similar. Sample 4 obtained using Protocol B has the lowest quality ($A_{260/280}$ = 2.94). Also average RNA concentration obtained from Protocol B was lower than Protocol C. The most prominent RNA was extracted using Protocol C in which average RNA concentration was at least 3 folds higher than the one obtained from Protocol B and D. The total RNA concentration extracted using Protocol D had also low yield and quality.

The integrity of total RNA was verified with Bioanalyzer 2100 (Agilent) microfluidic electrophoresis chips. Figure 1 shows gel-like images and electropherograms selected from four RNA samples with varying integrities. The RNA 6000 ladder contains six RNA fragments ranging in size from 0.2 to 6 kb (Mueller et al., 2006). The software automatically compares the unknown samples to the ladder fragments to identify the ribosomal RNA peaks. However, due to the low yield of RNA sample obtained from Protocol A, 18S and 28S ribosomal RNA peaks were not detected. Only lower marker, a spike-in control, was observed in this sample. Although electropherograms of samples obtained from Protocol B and C resembled each other, the differences arose from the integrity of total RNA. Partially degraded total RNA which showed a decrease in the 18S/28S ribosomal RNA band ratio, and an increase in fragmentation products, was observed in sample obtained using Protocol B. On the contrary, gel-like image and electropherograms of RNA from Protocol C showed a profile of intact and high quality total RNA. In the presence of contaminating genomic DNA, hills can be seen in the electropherograms. A distinctive hill and partially degraded RNA fragments were detected in RNA sample from Protocol D. According to Bioanalyzer electropherograms, total RNA obtained from Protocol C showed the highest quality and integrity when compared with all the other protocols. RNA concentration and quality results obtained with Bioanalyzer were consistent with results from AlphaSpect µL Spectrophotometer.

Amplification from RNA samples using LightCycler 1.5 Thermocycler

Amplification of the 18SrRNA gene, a housekeeping

Table 3. Concentrations (µg/µl) and values for ratio of OD recordings at 260 to 280 nm ($A_{260/280}$) of total RNA isolated using protocols A, B, C and D. Concentrations are indicated as mean ± S.E.M.

Protocol	Pretreatment	Sample 1		Sample 2		Sample 3		Sample 4	
		µg/µl	$A_{260/280}$	µg/µl	$A_{260/280}$	µg/µl	$A_{260/280}$	µg/µl	$A_{260/280}$
A	2×10^6cells no enzyme	0.05 ± 0.06	2.30	0.03 ± 0.04	2.31	0.02 ± 0.02	2.23	0.04 ± 0.05	2.26
B	2×10^6cells 0.5 mg/ml lyticase	1.46 ± 0.30	2.01	2.42 ± 0.24	1.98	2.47 ± 0.31	1.91	3.17 ± 1.29	2.94
C	6×10^7cells 5 mg/ml lyticase	7.76 ± 0.96	2.02	8.12 ± 0.40	2.03	7.94 ± 0.28	2.04	8.11 ± 0.82	2.04
D	2×10^6cells High Pure RNA isolation kit	0.41 ± 0.17	2.25	0.34 ± 0.08	2.25	0.48 ± 0.14	2.13	0.55 ± 0.05	2.14

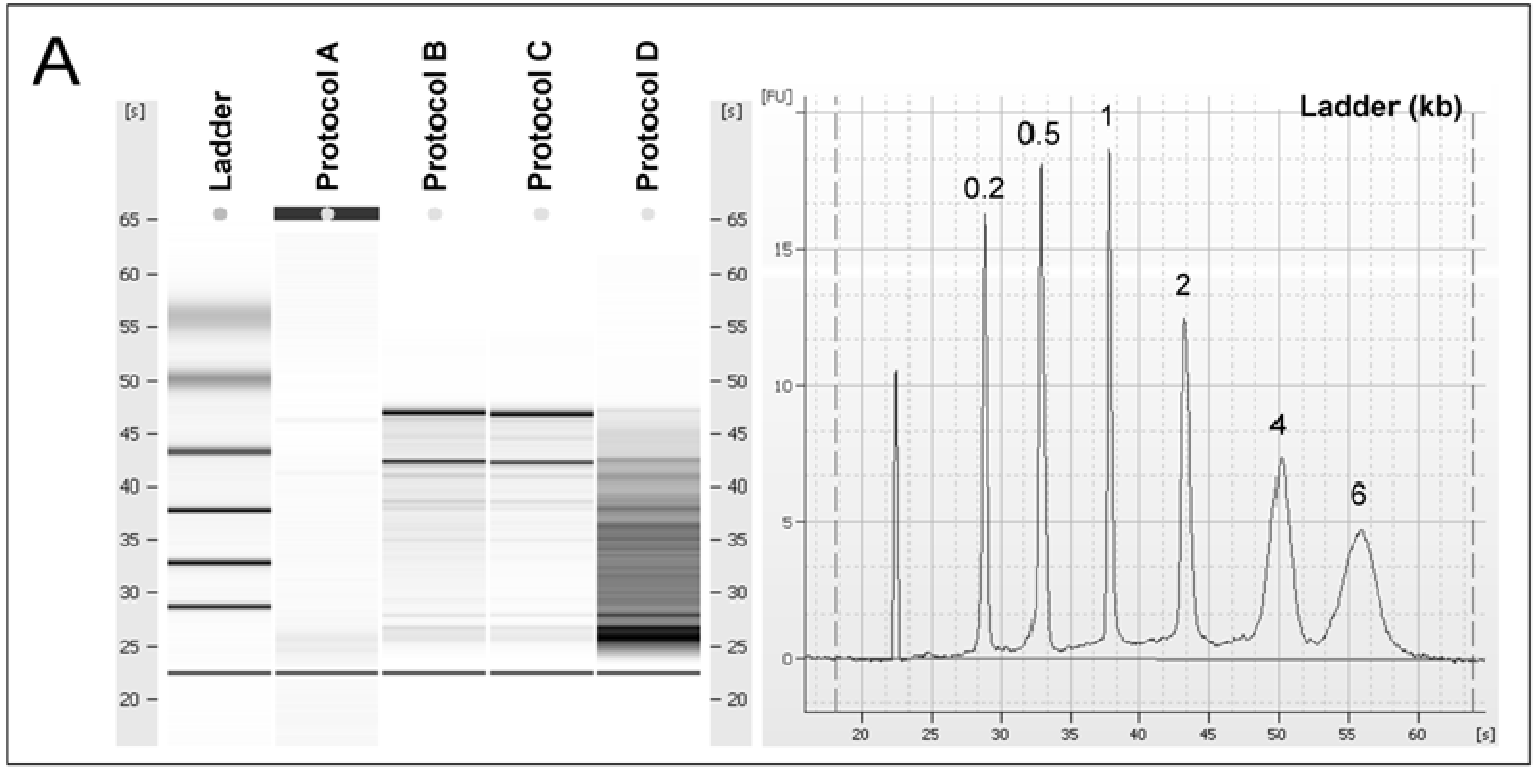


Figure 1. Gel-like image and electropherograms of RNA isolated using different protocols. Images were obtained from Agilent 2100 Bioanalyzer after an analysis run with RNA 6000 Nano LabChip. (A) The first lane shows RNA ladder (L), sample lanes 1 to 4 show yeast RNA isolated using Protocol A (1), Protocol B (2), Protocol C (3) and Protocol D (4). (B) The electropherograms of same samples display the intensity of each band on the gel. Arrows indicate 18S and 28S ribosomal bands.

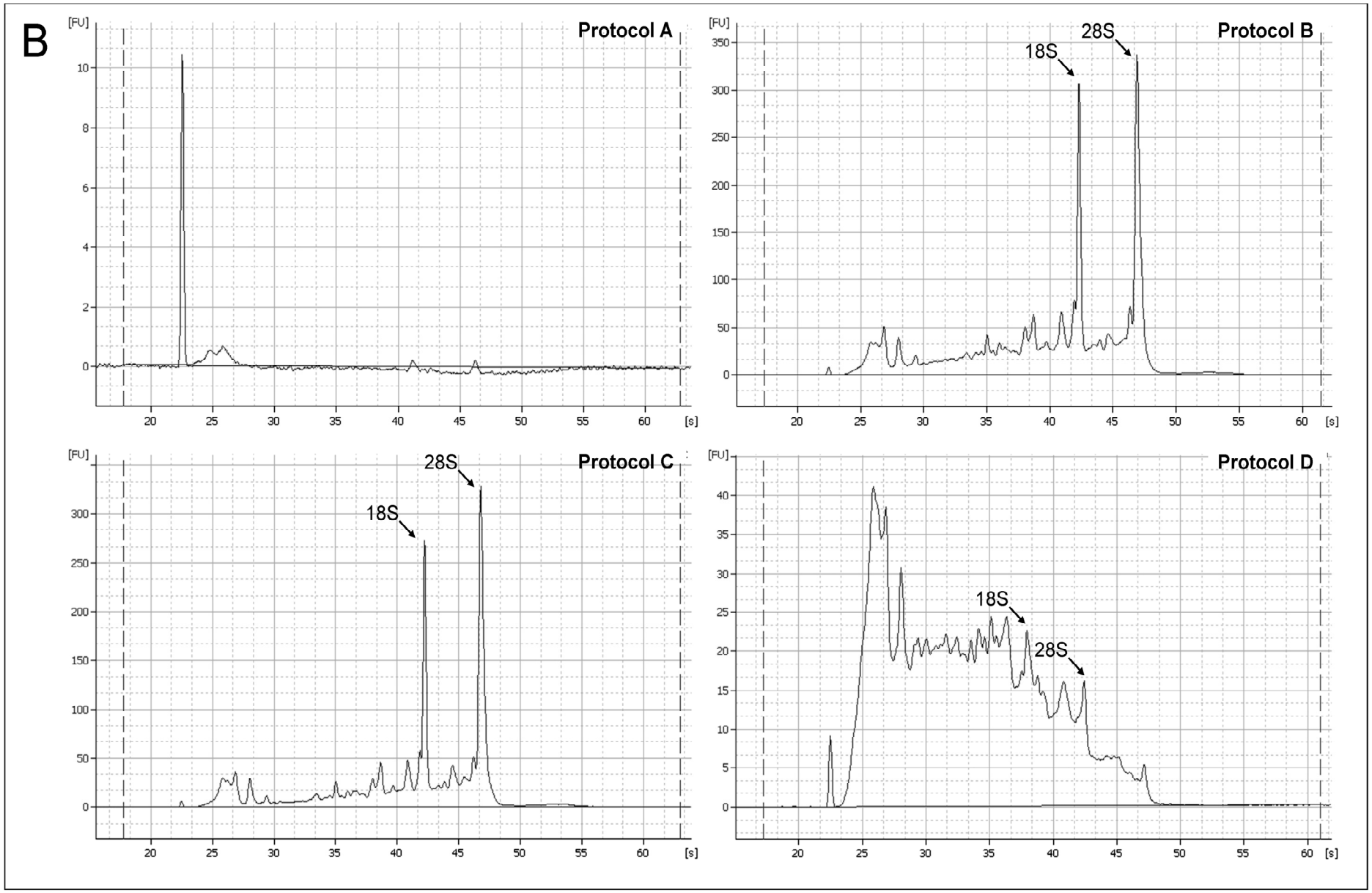


Figure 1. Contd.

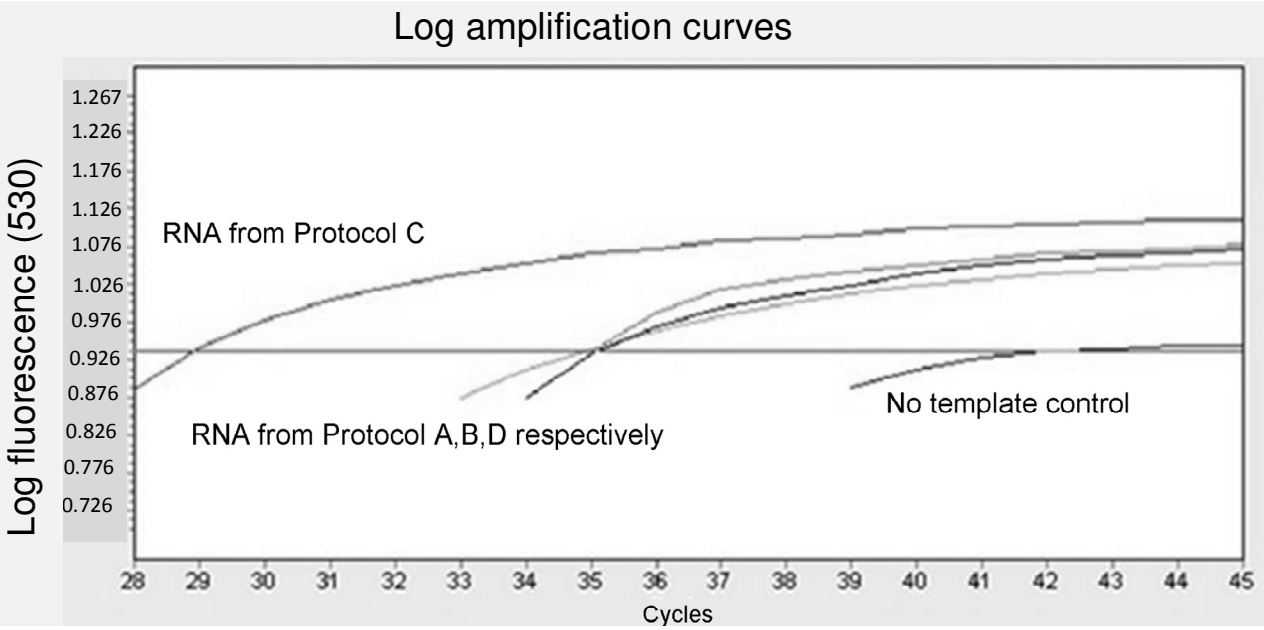


Figure 2. Log amplification curves of 18SrRNA gene measured with SybrGreen I using LightCycler 1.5 thermocycler.

gene, was performed using the LightCycler 1.5 with SYBR I assay. Log amplification curves are given in Figure 2. Threshold cycle numbers of cDNA samples synthesized from RNA extracted using Protocols A, B and D were 35.4 and Protocol C was 29.8. The sensitivity of the reaction with RNA from Protocol C was higher than the other samples from Protocols A, B, and D.

Quality assessments of Affymetrix GeneChip hybridizations

According to the 18SrRNA gene amplification results in qPCR, two samples from Protocol C were chosen for microarray hybridization. There are some quality control parameters in microarray associated with performance of the assay and hybridization to obtain highly reproducible gene chip probe array results. Evaluation of samples should be based on array performance metrics like probe array image inspection, B2 oligo performance, average background, noise values, percentage of present calls (% P) and signal from Poly A controls (lys, phe, thr, dap) and internal control genes (Yılmaz et al., 2008). In this study, probe array image inspections and B2 oligo performances of two array images indicated that there were no defects on the array. Moreover, expected altering patterns of intensities on the borders and the checkerboard pattern at each corner were observed and the array name was located in the upper-left corner of the array (Figure 3). The average background values of two arrays were 25.70 and 26.10. Noise mean values of array 1 and array 2 were 0.48 ± 0.04 and 0.47 ± 0.04, respectively. The entire poly A controls were called present with increasing signal values in the order of lys, phe, thr and dap for two arrays. Hybridization controls were used to evaluate sample hybridization efficiency. In both arrays, hybridization controls bioB, bioC, bioD and cre were always present. A set of internal control probe sets (AFFX-YFL039C, AFFX-YER148W, and AFFX-YER022W) can be used to monitor the sample quality at the hybridization step. Thus, 3'/5' signal ratios below 3.0 for housekeeping genes are expected values for good quality samples. Moreover, values such as % P are very good indicators of outlier samples or arrays within similar sample types and runs. Array 1 and array 2 showed a mean % P of 49.40 and 48.30, respectively. As a result, RNA isolated with Protocol C was suitable for use in a real-time qPCR and Affymetrix probe labeling reactions (Figure 4).

Actually, the goal of most microarray experiments is to survey patterns of gene expression by assaying the expression levels of thousands to tens of thousands of genes in a single assay. Affymetrix strategies include the use of a single label and independent arrays for each sample or for individual RNA preparations (Quackenbush, 2002). Expression values, computed from CEL files, were processed by RMA (Robust Multiarray Analysis), which is a model of normalization over multiple arrays, with GeneSpring GX 10.0 (Agilent) (Irizarry et al., 2003; Öz et al., 2009). RMA-normalized intensity values from each hybridization assays are shown in Figure 4. The normalization was performed in order to compare the genes in each of the biological samples. We obtained high-quality of signal intensities from both hybridizations for each gene element on the array.

Successful extraction of high quality total RNA from yeast cells should allow reliable and reproducible results

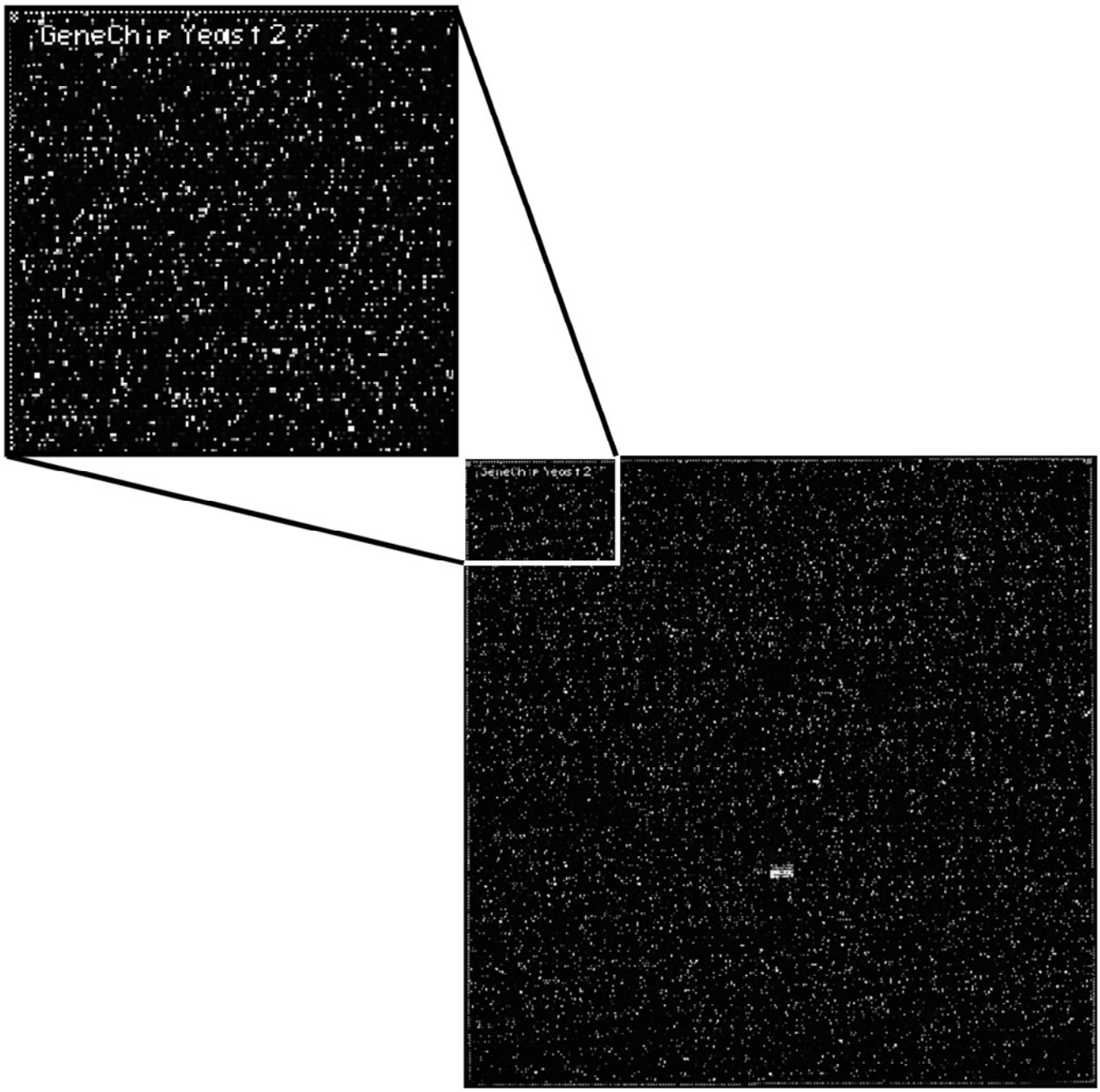


Figure 3. Microarray chip image of hybridization with sample from Protocol C.

in qPCR and microarray analysis. Many different techniques of varying success have been published (Rivas et al., 2001; Epstein and Butlow, 2003; Mannan et al., 2009). We found that the pretreatment applied in Protocol C which employs lysis of a high concentration of yeast cells and isolation of RNA produced higher RNA yields than other protocols. Protocol C was even more effective than the commercially available kit that was used in this study. The key points of pretreatment in extraction of yeast total RNA, as can be seen from Protocol C, were determined to be the starting amount of cell and enzyme concentration used in cell lysis to obtain maximum quality and yield of RNA. The merit of RNA for expression studies was checked by the LightCycler 1.5 with SYBR Green I assay. 18SrRNA gene was amplified using RNA from Protocol C with a high efficiency. Moreover, pretreatment in Protocol C produced sufficient total RNA for Affymetrix GeneChip hybridization assays, which resulted in gene expression data sets with acceptable quality metrics.

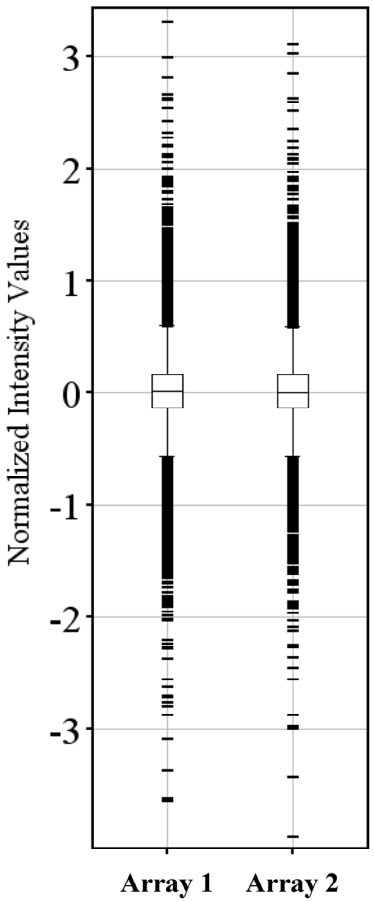


Figure 4. Normalized intensity values of two arrays.

In conclusion, isolated RNA using Protocol C is suitable for downstream applications including reverse transcription, real-time qPCR analysis and microarray hybridization without further processing. The protocol might also be suitable for northern blot analysis, cDNA library construction and rapid amplification of cDNA ends.

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