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# An effective and rapid method for RNA preparation from non-conventional yeast species

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ABSTRACT

The increased use of high-throughput RNA-based analysis has spurred the demand for rapid and simple preparation of high quality RNA. RNA preparation from non-conventional yeasts having diverse cell wall and morphological characteristics is often inefficient using current methods adapted for the model yeast, *Saccharomyces cerevisiae*. We report a simple RNA preparation method based on glass bead-mediated breakage in a formamide/EDTA solution. High quality RNA is generated within 15 min from various non-conventional yeasts species. The obtained RNA can be directly used for experimentation without further RNA purification and buffer exchange.

Advanced RNA-based analysis technologies, such as micro-array, RNA sequencing, and quantitative real-time polymerase chain reaction (qRT-PCR), require simple and rapid RNA preparation with minimal exposure to unintended conditions to obtain sufficient quantities of non-degraded, high quality RNA. *Saccharomyces cerevisiae* has been used as a model yeast to study many characteristics of eukaryotes [1], as an industrial microbe for many fermented beverages and bioethanol production [2], and as the host strain for the production of useful medicinal recombinant proteins and metabolites [3]. In recent decades, non-conventional yeasts, or non-*Saccharomyces* yeasts species, have drawn increased attention for diverse biotechnological applications [4]. Various non-conventional yeasts species are responsible for flavor development in fermented foods like traditional alcoholic beverages [5], and have unique characteristics, such as thermo/osmo-tolerance, that are suitable for sustainable bioprocesses, such as simultaneous saccharification and fermentation for bioethanol production [6]. Several non-conventional yeasts have been developed as a host system for production of metabolites and recombinant proteins with industrial potential [7]. Development of traditional molecular genetic techniques and more advanced synthetic biology tools in non-conventional yeasts species is expected to expand and diversify their impact on biotechnology [8]. For a better understanding of the unique genomic and functional characteristics of non-conventional yeasts species, whole-genome sequencing and transcriptome analysis have become essential to provide comprehensive information on physiological activities and regulation of gene expression in metabolic pathways with potential biotechnological importance. Comparative transcriptome profiling data under diverse culture conditions will facilitate the identification of key targets for metabolic engineering [9].

There are numerous genus and species of yeasts. They have very different morphological characteristics that include capsule structure of *Cryptococcus neoformans* [10], multi-polar hyphae of *Saccharomycopsis fibuligera* [11], and pseudo-hyphae of *Yarrowia lipolytica* [12]. Isolation of high quality total RNA from non-conventional yeasts with diverse cell wall structure and morphological characteristics is often inefficient using current methods and RNA extraction kits, which are adapted for *S. cerevisiae*. Current methods to obtain RNA from *S. cerevisiae* include acid hot-phenol extraction [13], a water bath method [14], the RNAsnap™ protocol [15], and one-step hot formamide extraction [16]. Although RNAs are efficiently obtained from *S. cerevisiae* using those methods, the preparation of high quality RNA is often challenging in other yeasts species that have mycelial formation with hyphae, thick cell wall, and carbohydrate capsule structure. For total RNA extraction from the multi-polar mycelial yeast *S. fibuligera* [11] and the encapsulated yeast *C. neoformans* [17], frozen yeast cells were ground in liquid nitrogen using a mortar and a pestle before total RNA extraction. However, grinding requires large amounts of cells and the RNA obtained from the same quantity of yeast cells can vary markedly due to the differences of physical force. We recently tried to prepare total RNA samples from the non-conventional dimorphic pseudo-hyphal forming yeast *Hyphopichia burtonii* [18] using several current RNA extraction methods, including hot-acid phenol, grinding-combined with RNA

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| Steps | Time (min) |
|---|---|
| Transfer yeast cells (2 $OD_{600}$ unit) to 1.7-ml microcentrifuge tubes and centrifuge at 13,000 rpm for 1 min | 3 |
| Wash the cell pellets with DW and remove the supernatant completely | 5 |
| Resuspend the cell pellets in 100 µl FE solution and add 50 µl of micro glass beads | 2 |
| Grind cells using a cell homogenizer by vortexing with 6,500 rpm for 90 sec at RT | 2 |
| Centrifuge at 13,000 rpm for 1 min and transfer 50~70 µl supernatant to a new tube | 3 |
| **Total process** | **~ 15** |

RNA samples in FE solution

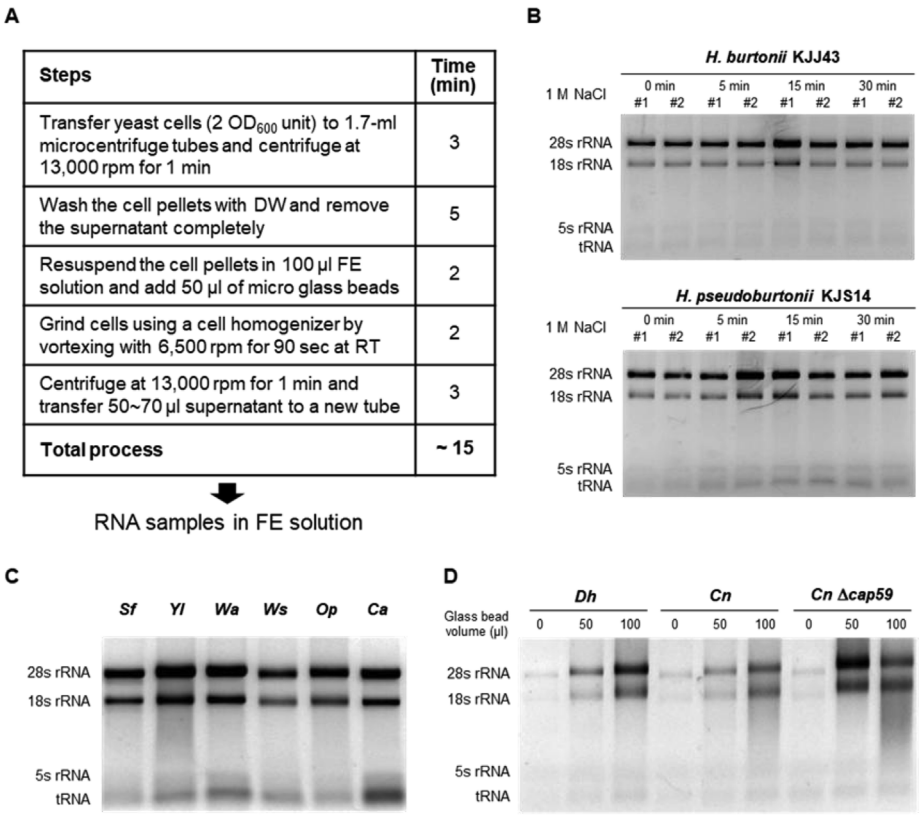


**Fig. 1. Yeast RNA extraction method based on FE/glass bead breakage.** (A) Outline of the FE/glass bead breakage method for small-scale RNA extraction from yeast cells. (B) Gel analysis of RNA samples in FE solution. *H. burtonii* and *H. pseudoburtonii* were cultivated in YPD with 1 M NaCl for the indicated times (0–30 min) to impose an osmotic stress. RNAs were extracted from the yeast cells with total $OD_{600}$ 2.0 and equal volumes of each samples were separated by 1.2% agarose MOPS/formaldehyde gel electrophoresis. (C) RNA sample preparation from various non-conventional yeasts species. *Sf*: *S. fibuligera*, *Yl*: *Y. lipolytica*, *Wa*: *W. anomalus*, *Ws*: *W. subpelliculosus*, *Op*: *O. parapolymorpha*, and *Ca*: *C. albicans*. Yeast cells were cultivated in YPD and total RNAs were extracted (total $OD_{600}$ 2.0). (D) Optimization of glass bead volume. *Dh*: *D. hansenii*, *Cn*: *C. neoformans*, *Cn Δcap59*: *C. neoformans* acapsulular strain. Different volumes of glass beads (0, 50, 100 µl) were added to the yeast cells ($OD_{600}$ 2.0) suspended in 100 µl FE solution. RNA samples in FE solution were directly mixed with 2 x RNA sample loading buffer (R1386-1VL, Sigma-Aldrich) and SafePinky nucleotide staining solution (S1001-025, GenDEPOT).

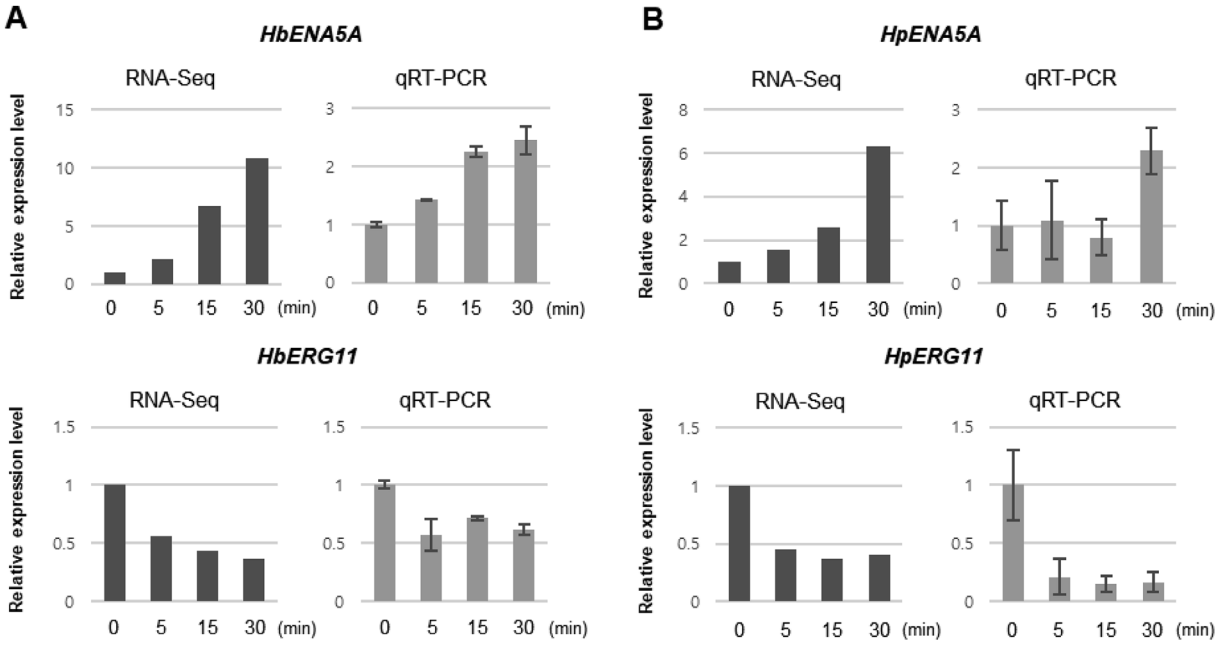


**Fig. 2. Application of the RNA samples prepared by FE/glass bead breakage to downstream enzymatic reactions.** Relative expression levels of *H. burtonii* and *H. pseudoburtonii* genes (*ENA5A* and *ERG11*) upon 1 M NaCl treatment, as analyzed by qRT-PCR and RNA-Seq. The expression levels of genes by RNA-Seq were quantified using TopHat and Cufflinks [24] with the value of fragments per kilobase of exon per million fragments mapped (FPKM), and differential expressions between control (0 min) and other conditions (5, 15, or 30 min) were analyzed using Cuffdiff with two replicates with the cutoff at p-value $< 0.01$. For qRT-PCR, each sample was analyzed in duplicate and normalized by endogenous control genes *HbATP4* and *HpCYS3,* respectively, which showed no apparent expression changes at the transcript level in the presence of 1 M NaCl. The amplification efficiency of the primers of targets and control genes used for qRT-PCR was validated very similar by analyzing $\Delta C_T$ values with serially diluted cDNA.

isolation kit (Qiagen RNeasy Mini Kit, 74104), and one-step hot formamide extraction. We failed to obtain high quality RNA (see Supporting Fig. S1 A, B, and C in the supplementary material).

Here, we report a simple RNA extraction method based on glass bead-mediated breakage in formamide/ethylenediaminetetraacetic acid (EDTA) solution (FE) at room temperature (RT) (Fig. 1A). The procedure is performed in microcentrifuge tubes and takes only 15 min.

## 1. Protocol

For RNA sample preparation, yeast cells are cultivated overnight in 2–3 ml of yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone and 2% glucose) at 28 °C with shaking at 220 rpm. The pre-cultured yeast cells are inoculated in fresh YPD medium at an optical density at 600 nm ($OD_{600}$) of 0.3–0.6, grown to an $OD_{600}$ of approximately 1.0–2.0, which is nearly the early phase of exponential growth.

Yeast cells (total 2 $OD_{600}$ unit) are transferred to 1.7 ml microcentrifuge tubes and harvested (13,000 rpm, 1 min, RT) using a tabletop microcentrifuge. The supernatant is completely removed by pipetting (an important step, since residual can affect the efficiency and quality of the RNA extract) and the cells are washed by resuspension in 1 ml of distilled water (DW) and centrifugation. The washed cells can be stored at −80 °C after freezing with liquid nitrogen. When required, the cell pellet is suspended in 100 µl of FE (98% formamide, 0.01 M EDTA), prepared by mixing formamide (99.5%, F9037; Sigma-Aldrich) and EDTA (0.5 M, pH 8.0, ML005-01; WelGENE). For optimal results, the FE solution volume is increased in proportion to the volume of the cell pellet (50 µl per 1.0 $OD_{600}$ of cell suspension). A 50 µl volume of RNase-free glass beads (500 µm, GB05-RNA; Nextadvance) is added and the cell suspension is ground by vortexing for three cycles of 30 s (total 90 s) at 6500 rpm at RT using a Pre-cellys 24 homogenizer (Bertin Technologies). If a different homogenizer is used, such as an MT-360 TOMY (Seiko Corporation), the vortex time may need to be optimized (see Supporting Fig. S1D, Fig. S1E, and Fig. S2). The optimal vortexing time was defined as the shortest time generating the maximum quantity of RNA with the 28S:18S ratio above 1.8, which was chosen as 90 s with Pre-cellys 24 homogenizer in our study. The homogenized sample is centrifuged at 13,000 rpm for 1 min at RT. The supernatant is transferred to a new 1.7 ml microcentrifuge tube. The extracted but unpurified RNA in this FE solution can be directly assessed by RNA gel electrophoresis in 10% 3-morpholinopropane-1-sulfonic acid (MOPS)/0.75% formaldehyde running buffer. This RNA can also be directly used for sequential DNase treatment or cDNA synthesis reaction. To avoid interference of the enzyme reaction by formamide, it is critical to dilute the RNA samples in FE solution to less than 5% of the total solution [19]. If the RNA concentration in FE solution is higher than 100 ng/µl, the RNA sample can be directly used for RNA-Seq analysis without any further treatment. But, when the RNA concentration is not high enough, ethanol precipitation is recommended to concentrate RNA. As an option, the RNA in FE solution can be diluted in diethyl pyrocarbonate (DEPC)-treated water (C-9030; Bioneer). The sample can be stored at −20 °C or −80 °C at this step.

The method yielded high quality RNA samples from the mycelial yeast *H. burtonii* KJJ43 and *H. pseudoburtonii* KJS14 isolated from Korean Nuruk fermentation starter (Supporting Table S1). The RNA samples in FE solution were directly subjected to RNA formaldehyde-agarose gel electrophoresis, which revealed non-degraded large rRNA and low molecular weight tRNA and 5S rRNA (Fig. 1B). We further applied this method to the preparation of RNA samples from various non-conventional yeasts species, including *S. fibuligera* KJJ81, *Y. lipolytica* PolF, *Wickerhamomyces anomalus* Y4, *Wickerhamomyces subpelliculosus* SMY-04, *Ogataea parapolymorpha* DL1-L, *Candida albicans* ATCC32354, *Debaryomyces hansenii* KD-2, *Cryptococcus neoformans* H99, and *C. neoformans* acapsular mutant Δ*cap59* (Fig. 1C and D). In the case of *D. hansenii*, which forms biofilms [20], and *C. neoformans*, which possesses a thick extracellular capsule, we tested different volumes of glass beads. The use of 50 µl was generally appropriate for the preparation of RNAs from the most yeasts species tested in this study. An increased amount of glass beads could produce more efficient cell lysis, but could result in more degradation of RNA. Compared to the wild type *C. neoformans* strain, RNA was extracted more efficiently from the acapsular strain *C. neoformans* Δ*cap59*, indicating that the capsule structure might lessen yeast cell breakage by glass beads.

The quality of the RNA samples of *H. burtonii* and *H. pseudoburtonii* cultivated with 1 M NaCl to impose osmotic stress was further assessed by several quality control analyses after ethanol precipitation purification of the RNA samples in FE solution. The concentration and RNA integrity number (RIN) of RNA samples prepared from small-scale yeast cell suspensions (total $OD_{600}$ 2.0) were sufficient for RNA-Seq analysis (Supporting Tables S2A and B). In the case of *H. burtonii,* the final yields of total RNA from the same total cell OD were variable among the samples, due to the physiological change of this hyphae forming yeast under salt stress conditions. Despite such differences in final yields among the RNA samples from *H. burtonii* cells cultivated under different osmotic conditions, such as cultivation in the presence of 1 M NaCl, 1 M KCl, and 1 M sorbitol, the quality of RNA samples prepared by FE/glass bead breakage method was mostly good enough for RNA-Seq analysis with high ratio of 28S/18S and RIN values (Supporting Table S3). For RNA-Seq analysis, the qualified RNA (1 µg) in each sample was subjected to poly(A) mRNA enrichment by using magnetic beads with oligo (dT) and then sheared into short fragments. Using reverse transcriptase and random hexamer primers, the first strand cDNA of mRNA fragments was synthesized, and the second strand cDNA was then synthesized using DNA Polymerase I and RNase H. The synthesized cDNA was subjected to end-repair and poly(A) tailing and connected with sequencing adapters using a TruSeq Stranded mRNA Sample Prep Kit (Illumina). The proper cDNA fragments, purified by a BluePippin instrument (Sage Science), according to the manufacturer's instructions, were selected for further PCR amplification. Subsequently, the libraries were subjected to paired-end sequencing with a 100 bp read length using an Illumina HiSeq 2500 platform. The feasibility of the RNA samples of *H. burtonii* and *H. pseudoburtonii* for polymerase chain reaction (PCR) analysis was assessed. The genes chosen for PCR were *H. burtonii ATP4* (*HbATP4*), encoding a mitochondrial ATP synthase subunit, and *H. pseudoburtonii CYS3* (*HpCYS3*), which codes for cystathionine gamma-lyase in the cysteine assimilation pathway. These genes were chosen because they were shown to be expressed constitutively regardless of salt stress conditions in our RNA-Seq data (GEO submission number GSE130141). The sequences of PCR primers are presented in Supporting Table S4. When we conducted PCR with the primer sets of *HbATP4* and *HpCYS3* using the RNA samples in FE solution as templates, we did not observe any DNA bands amplified by PCR before cDNA synthesis (Supporting Figs. S3A and B). The absence of amplification of PCR products from the RNA samples without DNase treatment indicated the lack of contamination by chromosomal DNA of the RNA prepared by the FE/glass bead breakage method. For cDNA synthesis, the RNA samples in FE solution were used directly without buffer exchange and DNase treatment. After cDNA synthesis, we confirmed the amplification of *HbATP4* and *HpCYS3* DNA fragments by PCR from the synthesized cDNAs (Supporting Figs. S3C and D).

To examine the consistency of expression patterns of *H. burtonii* and *H. pseudoburtonii* genes between RNA-Seq data and other RNA analysis data, we carried out qRT-PCR analysis with the gene specific primers for *ENA5A* (encoding an ATPase sodium pump) and *ERG11* (encoding lanosterol 14-alpha-demethylase in the ergosterol biosynthesis pathway) of *H. burtonii* and *H. pseudoburtonii,* using the synthesized cDNAs as templates (Fig. 2). The activity of the sodium pump family is important for osmotolerance [21], and the expression levels of genes involved in the ergosterol pathway are decreased by osmotic stress in *S. cerevisiae* [22]. Our RNA-Seq data revealed the increased expression of *ENA5A* and the decreased expression of *ERG11* in both *H. burtonii* and

*H. pseudoburtonii,* as expected. For cDNA synthesis, 1 μl of RNA in FE buffer was diluted with 15 μl of DEPC-treated water, mixed with 4 μl of SuperiorScript III Master Mix (Enzynomics) for cDNA synthesis reaction at 42 °C for 45 min. The concentration of synthesized cDNA was adjusted to have Cq values within the range of 20–35 in qRT-PCR, which was carried out with CFX96 Optical Module (Biorad) using TB Green Premix Ex Taq (Takara). Gene expression values were calculated by the $2^{-\Delta\Delta CT}$ method [23] using *HpCYS3* and *HbATP4* as internal control, respectively, for *H. burtonii* and *H. pseudoburtonii* genes in duplicated experiments. The relative expression patterns of *ENA5A* and *ERG11* detected by qRT-PCR were consistent with those based on RNA-Seq data, although the fold change values were generally lower in the qRT-PCR data compared to those in the RNA-Seq data (Fig. 2A and B), indicating that this method ensures good quality of RNA samples, which is important for experimentally reliable and repeatable results.

The RNA preparation method presented in this paper is a rapid (within 15 min) and simple procedure that can be done at RT. Compared to the widely used hot-acid phenol method and the recently developed one-step hot formamide extraction method, this method does not employ hot temperature and takes much less time to obtain high quality RNA samples (Supporting Fig. S1F). With this method, a very small amount of cell mass ($OD_{600}$ 2.0) is sufficient to generate RNA samples required for downstream applications, such as RNA-Seq and qRT-PCR analysis. The method can be easily adapted for high-throughput analysis of multiple RNA samples generated from diverse yeasts species. With the increasing attention on non-conventional yeasts species in both academic and industrial sectors, we anticipate that our RNA isolation method will be useful for diverse RNA-based analyses for a variety of yeasts species having diverse morphologies and cell wall structures.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2019.113408.

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