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RNA Extraction from the Yeast *Candida parapsilosis* sensu stricto Using Two Commercially Available Silica Column-Based Purification Methods

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Abstract

Good quality ribonucleic acid (RNA) needs to be obtained in order to study gene expression. Different RNA extraction methods have been described, but RNA quality and yield may vary among the different techniques and biological study species. To date, there is no standardized method for extraction and purification of RNA from *Candida* genus yeasts. The few available papers on the subject apply mainly to filamentous fungi and have produced poor results for extraction techniques based on manual methods. The aim of this study was therefore to compare two commercial RNA extraction and purification systems using silica columns (Qiagen and Zymo Research) with *Candida parapsilosis* sensu stricto as model organism. This yeast has been identified in recent papers as the second most frequently isolated *Candida* species in the oral cavity. In the past decade, it has been the object of increasing medical interest because it is one of the main causes of candidemia in both adults and preterm neonates. In view of this background, we consider the study of *Candida parapsilosis* sensu stricto transcriptome and its variations according to environmental changes to be a priority. In this experimental study, 19 fungal isolates were processed using Qiagen and 17 isolates using Zymo Research. The results suggest that Qiagen lysis buffer RLT is essential for obtaining better quality RNA product.

Keywords: RNA Extraction; RNA Purification; *Candida parapsilosis* sensu stricto; Silica Columns

Introduction

Developments in molecular biology have enabled molecular techniques to be used in mycological studies, thereby promoting more precise diagnoses in shorter times. Moreover, techniques for analysis of gene expression have enabled the study of gene function, providing understanding of interactions between the host and its mycobiome, and the response of each fungal species to different environmental conditions.

Candida parapsilosis sensu stricto is part of the human mycobiome, and according to two studies on oral mycobiome, is the second most frequently isolated *Candida* species in the oral cavity [1,2]. In the past decade, it has been the object of increasing medical interest because it is one of the main causes of candidemia, especially in Latin America, Europe and Asia; in both adults and preterm neonates [3-7]. Considering this background, it is a priority to study the transcriptome of this *Candida* species and its variations in response to environmental changes.

Good quality (ribonucleic acid) RNA is needed to study gene expression. It is therefore important to use the best possible RNA extraction method, since any contaminants such as RNases, proteins, polysaccharides and genomic DNA (deoxyribonucleic acid) may affect quality of this biomolecule, and reduce the efficiency of its amplification. In addition, RNA is highly labile and less stable than DNA [8].

RNA extraction is particularly critical for fungal cells because their cell wall characteristics differ according to genus, and the processes must be optimized for each particular case. Different methods are currently available for disruption and homogenization of tissues with liquid nitrogen, sand, beads or mycelium lyophilization for filamentous fungi [9,10]. Techniques based on enzymatic disruption of the wall by zymolyase or lyticase are the most frequently used options for yeasts [11]. However, regardless of the method used, there is always a latent risk of rehydration of samples and activation of RNases. To date, different processes have been reported for RNA extraction, including among others, the use of phenolic compounds, triazoles, sodium dodecyl sulfate (SDS), lithium chloride, detergents such as hexadecyltrimethylammonium bromide (CTAB), and increasingly frequently, commercial extraction kits [12,13]. Nevertheless, the quality and yield of the extracted RNA may vary according to the methodology applied and the biological study species [14].

There is no standardized methodology to date for extraction and purification of RNA from yeasts of the genus *Candida*. The few papers on the subject apply mainly to filamentous fungi, and have provided poor results for extraction techniques based on manual methods. The aim of this study was therefore to compare two commercial systems, Qiagen and Zymo Research, for extraction and purification of RNA with silica columns. The main differences between the two systems are the homogenization system used for cell suspension and cost.

The Qiagen system was selected for its lysis buffer RLT, which has high concentrations of guanidine isothiocyanate and lacks phenol in its composition. As far as we know, there is no other system on the market with similar composition. The Zymo-research system was selected due to its lysis buffer based on a combination of phenol and guanidine, with the commercial name of Tri-reagent, which is equivalent to TRIzol®. These are two ideal systems to be compared in terms of their efficiency in obtaining good quantity and quality of RNA.

Material and Methods

Experimental Design

A retrospective, cross-sectional, comparative basic research study was designed to compare two commercial systems for extraction and purification of RNA with silica columns regarding their ability to provide more and better quality biomolecules from the fungal cell biomass of the yeast *Candida parapsilosis* sensu stricto. A collection of 36 isolates was used, which had been characterized as *Candida parapsilosis* sensu stricto in a previous study using molecular methods. The strains were assigned randomly for processing with the Qiagen system (n=19) or Zymo Research system (n=17). The research design of this study was approved by the Ethics Committee of the Faculty of Dentistry of the University of Buenos Aires (UBA), with file number 0048223/2016, and code 012/2016 CETICAFOUBA.

The MIQE guidelines proposed by Bustin *et al.* for the publication of experiments based on the study of gene expression were followed [15].

Fungal Isolation and Preservation

The *Candida parapsilosis* sensu stricto yeast isolates were obtained from the yeast collection at the Mycology Center of the Buenos Aires University School of Medicine. The fungus was previously selected on CHROMagar *Candida* (Becton-Dickinson) differential medium as *Candida parapsilosis* complex. This was followed by microscopic study on milk-Tween 80 agar and automated Vitek2 system. The species was confirmed by endpoint PCR using specific primers that join to the region ITS1-5.8SrRNA-ITS2, enabling identification of this particular species [16]. Each strain was kept for short periods on Sabouraud agar at 4 ˚C, and preserved for long periods in glycerol at -70 ˚C.

Culture Media

Several culture media were used, beginning with differential chromogenic (CHROMagar *Candida*) for 24 hours at 37 ˚C for initial selection. This was followed by Sabouraud medium for 24 hours at 28 ˚C to obtain a subculture and for strain maintenance. Finally, it was placed in YPD broth (yeast extract-peptone-dextrose) for 18 hours at 37 ˚C to obtain a more pure and enriched culture in exponential phase [17].

Sample Processing and Preparation of Spheroplast Suspension

To prepare spheroplasts, 20 ml of the culture in exponential phase in YPD broth was pelleted by centrifuging at room temperature at 3000 rpm for 5 minutes. The pellet was re-suspended in 1mL cold sorbitol, and the suspension was diluted with sorbitol at ratios of 1:2 and 1:3. The cell density in these dilutions was measured in a spectrophotometer in order to select the concentration that would provide highest RNA yield. The suspension obtained was pelleted twice in refrigerated microcentrifuge at 1000 x g for 5 minutes at 4 ˚C.

The pellet obtained from the second rinse was re-suspended in 100uL of spheroplast-forming solution (sorbitol+EDTA+beta-mercaptoethanol+ultra pure nuclease-free water+1.43mg/mL zymolase and incubated in a laboratory water bath at 37 ˚C for 2

hours (Table 5) [18]. Spheroplasts were verified by smear slides stained with toluidine blue and viewed under optical microscope (o.m) (Figures 1 and 2).

This step can be followed immediately by extraction and passage through the column, or the process may be deferred and the spheroplasts kept in a freezer at -80 ˚C [19].

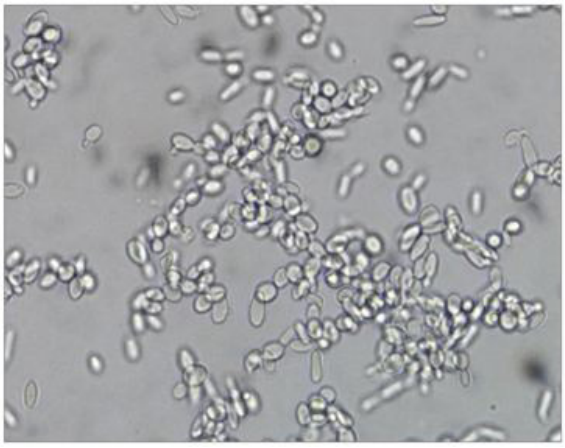


Figure 1: Yeasts with preserved cell wall, prior to the action of zymolase. (Unstained sample, 40X o.m.)

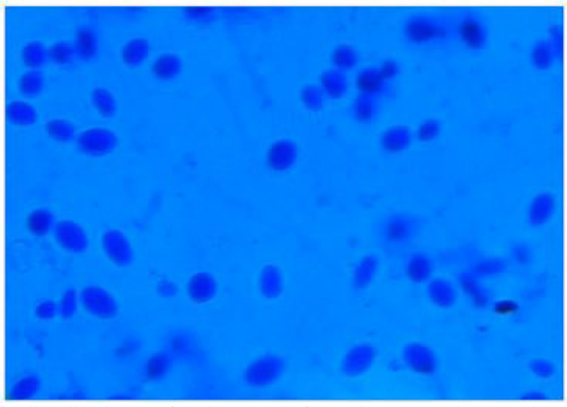


Figure 2: Yeast with altered cell wall, 2 hours after the action of zymolase. (Sample stained with toluidine blue, 40X o.m.)

RNA Extraction and Purification using RNeasy Mini Kit (Qiagen)+DNaseI System

The manufacturer's instructions to extract and purify RNA with the Qiagen system, from the fungal lysate, were followed. (See supplementary material 1). It should be noted that this system homogenizes the initial sample (spheroplast suspension) with guanidine isothiocyanate at high concentrations (Buffer RLT).

The treatment with DNase I (Qiagen brand) is subsequent to the addition of ethanol and prior to the rinses with the respective buffers. Anyway, manufacturer's instructions were followed for this step of the purification. Deoxyribonuclease I (Usually called DNase I), is an endonuclease coded by the human gene DNASE1. DNase I is a nuclease that cleaves DNA preferentially at phosphodiester linkages adjacent to a pyrimidine nucleotide, yielding 5'-phosphate-terminated polynucleotides with a free hydroxyl group on position 3', on average producing tetranucleotides. It acts on single-stranded DNA, double-stranded DNA, and chromatin [20,21].This step is essential to obtain better quality extracts [22].

The RNA obtained was re-suspended in 30 µL of nuclease-free water (Provided by Qiagen kit), and passed twice through the column.

RNA Extraction and Purification using Zymo-Research+DNase I System

The manufacturer's instructions to extract and purify RNA with the Zymo-research system, from the fungal lysate, were followed. (See supplementary material 2). It should be noted that this system homogenizes the initial sample (Spheroplast suspension) with a compound called Tri-reagent (Phenol+guanidine isothiocyanate).

The treatment with DNase I (Provided by the Zymo Research kit) is after adding ethanol and prior to rinses with the respective buffers. Similarly, manufacturer's instructions were followed for this step of purification.

The RNA obtained was re-suspended in 30 µL of nuclease-free water (Provided by the Zymo Research kit), and passed twice through the column.

Analysis of Yield, Purity and Integrity of RNA

The RNA extracts obtained were quantified using a Multiskan GO 10040/1510-02746C (Thermofisher) spectrophotometer, with wavelength 260 nm. The concentration (ng/uL) was obtained through of the following formula: Absorbance 260*40*(10/0,51).

RNA purity was determined for each sample through of the absorbance ratio 260/280, which measures contamination by proteins; and absorbance ratio 260/230, which measures contamination by carbohydrates, phenols and salts.

The results for purity and yield were processed and analyzed in Microsoft Excel 2010 and Info Stat 2018 statistical package, using mean, standard deviation and coefficient of variation. A 95% confidence interval was used and statistical significance was determined by bilateral Mann Whitney test because the data do not follow normal distribution. The data were represented in bar charts with error bars.

In addition, RNA integrity and contamination by genomic DNA were checked and recorded by direct visualization in 2% agarose gel stained with ethidium bromide. The gel was run at 93 volts for 1 hour and viewed in a UV transilluminator.

For indications of the manufacturer of each commercial kit, the RNA was conserved at -70 ˚C.

Preparation of Complementary DNA or DNA Copy (cDNA)

BioRad reverse transcriptase (iScript™ cDNA Synthesis Kit, 100 x 20 µl rxns #1708891), which was used to transcribe RNA to cDNA, is a genetically engineered MMLV.

The kit provides 400µL of the 5x reaction mix (Buffer+primers+stabilizers+deoxinucleotides); 100µL of iScript reverse transcriptase; and 1.5mL of nuclease-free water.

The reaction was prepared in a total volume of 20µL, constituted of 4µL reaction mix plus 1µL enzyme, The reaction was prepared in a total volume of 20µL, constituted of 4µL reaction mix plus 1µL enzyme, plus nuclease-free water as required, and RNA in an amount adjustable to a concentration of 1µg in the 20µL reaction volume. The manufacturer's instructions were followed for cycling conditions of the retrotranscription (BioRad Kit).

Design of Primers for Amplification of the ITS Region

With the aim of evaluating the quality of the RNA obtained, a set of primers was designed based on the ITS (Internal transcribed spacer) region, which is a sequence that transcribes without modifications and is present in all fungi, and used regularly for typing and genotyping (Figure 3). The sequence of the ITS region of a reference strain of *Candida parapsilosis* sensu stricto (ATCC 22019) was used, and PrimerBLAST software used to design the primers with parameters preset by the program. To determine specificity, a search was done in RefSeq genome databases for the organism *Candida*.

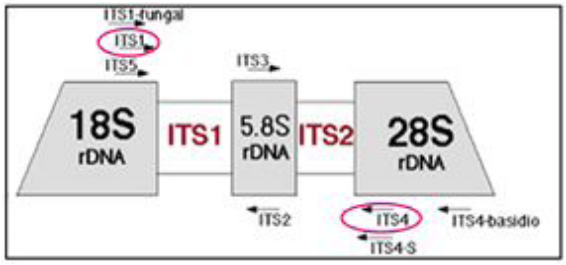


Figure 3: The ITS1 and ITS4 primers allow raising a region of the fungal ribosomal RNA of 517bp

The sequences obtained for the primers were the following: a) ITS1 forward: TCCGTAGGTGAACCTGCGG, and b) ITS4 reverse: TCTTTTCCTCCGCTTATTGATATG.

The primers were validated in silico and experimentally. The in silico analysis was carried out by PrimerBLAST algorithm, with which the primers were designed and the specificity verified by the existence or not of homology with the ITS1-ITS2 region of the ribosomal DNA.

Experimental validation of primers was done by RT-PCR, using the cDNA of the set of samples selected; and sequencing of the purified amplification product plus bioinformatic analysis for Nucleotide BLAST [(Basic Local Alignment Search Tool), (https://blast.ncbi.nlm.nih.gov/Blast.cgi)]. This analysis was carried out with the objective of confirming the specificity of the primers, by demonstrating the homology of the PCR product with the ITS1-ITS2 regions of ribosomal DNA.

Conditions and Performance of the RT-PCR

To do the RT-PCR, the RNA obtained was transcribed to cDNA (DNA copy) with the BioRad kit (iScript cDNA Synthesis kit 100x20µL reactions), according to the manufacturer's instructions, based on 1µg RNA altogether. Using the cDNA obtained, the

RT-PCR was performed under the following conditions: 8 cDNA samples processed by Qiagen and 8 cDNA samples processed with the Zymo-Research system were selected at random, to be subjected to RT-PCR of the region ITS1-ITS2, using the pair of primers described above. The PCR was performed with 3 controls: a positive control based on genomic DNA from a reference strain for C. parapsilosis sensu stricto ATCC 22019; a negative control based on replacing cDNA by water, and a detection limit control based on cDNA diluted 1:10. The unknown cDNAs were diluted 3:10. The final concentration and volume of each component of the PCR per tube were as follows: Water 9.85; PCR buffer (1X), 2.5 uL; Cl2Mg (3 mM) 1.5 uL; dNTPs (0.2 mM), 0.5 uL; Primer ITS1 (0.4 uM), 0.2 uL; Primer ITS 4 (0.4 uM), 0.2 uL; Taq-polimerase (1.25U/uL), 0.25 uL; and cDNA 10 uL. The PCR cycles were performed in a MiniCyclerTM, MJ Research INC thermal cycler, under the following protocol: one 5-minute cycle at 95 ˚C, followed by 30 cycles of 3 stages (20 seconds at 95 ˚C //15 seconds at 55 ˚C // 65 seconds at 72 ˚C), and finally, one 5-minute cycle at 72 ˚C.

Statistical Analysis

The data on concentration and purity, obtained by each commercial system, were processed and analyzed in the Microsoft Excel 2010 programs, and the statistical package Info Stat 2018. For the descriptive analysis of the variables, the average, standard error, and median were used. The data was represented in tables and graphs of columns with error bars.

The normality of the data was established by the Q-Q-plot test and the Shapiro Wilks test (Data not revealed). On the other hand, the homogeneity of variances was determined for each of the variables studied using the Fisher variances quotient test. The detection of outliers was performed by Grubbs test.

With respect to statistical inference, both parametric tests (Test t for two independent samples) and non-parametric tests (Mann-Whitney U test for two independent samples); considering a value of p less than alpha error, assuming as alpha a value equal to 5%.

Repeatability and Reproducibility of Each Commercial Kit

The precision of each system was determined by repeatability and reproducibility. Repeatability was evaluated by assaying 4 replications of the same sample or strain at the same time (day 1), calculating average, standard deviation and coefficient of variation. Reproducibility was determined by assaying 4 replications of the same sample or strain on a different day (day 2), calculating average, standard deviation and coefficient of variation of the 4 matrices and contrasting with day 1 results. Inter-operator variability was evaluated by independent analysis of 8 replications of the same sample or strain done on the same day by two different operators from the same laboratory (4+4). Average, standard deviation and coefficient of variation were calculated. This procedure was done for each kit or commercial system.

Results and Discussion

Comparison of Qiagen and Zymo Research Systems

Results for concentration and purity were compared (Table 1). To detect differences between the means of RNA concentration obtained with both commercial systems, Student's t test was used for two independent samples, since both variables demonstrated normality and equality of variances and absence of outliers; hypothesizing that the difference between the means is not equal to. Figure 4 shows that RNA yield was significantly greater for the Zymo Research system than the Qiagen system (p=0.00054). This difference was probably caused by the Zymo Research system's guanidine phenol-isothiocyanate-based compound used for homogenization, since the Zymo Research and Qiagen protocols are very similar. It is likely that phenol in combination with guanidine isothiocyanate synergize by enhancing the lytic effect, since phenol acts as a protein denaturing agent; and guanidine as a lytic agent [23,24]. This undoubtedly impacts performance, since the greater the break, the greater the amount of nucleic acids that remain available in the supernatant. Indeed, a study by Sandoval *et al.* evaluating different methods (TRIzol, CTAB+ LiCl2 and RNeasy Mini kit by Qiagen) for extraction of RNA from the native fungus Xylaria sp. found that the differences in RNA yield were mainly affected by the homogenization system and protocol used [25]. To date, no study on the subject or comparing two commercial systems has been published specifically for yeasts.

Variable	QIAGEN SYSTEM			ZYMO-RESEARCH SYSTEM		
	Mean	S.E	95%CI(Mean)	Mean	S.E	95%CI(Mean)
RNA (ug/uL)	1,3	0,15	0,99-1,61	2,08	0,14	1,78-2,38
Abs 260/280	2,04	0,03	1,99-2,1	1,83	0,06	1,71-1,95
Abs 260/230	1,53	0,15	1,23-1,84	1,99	0,07	1,83-2,14

Note: S.E= standard error.

Table 1: Descriptive and inferential statistics for the yield and purity variables with each commercial system tested

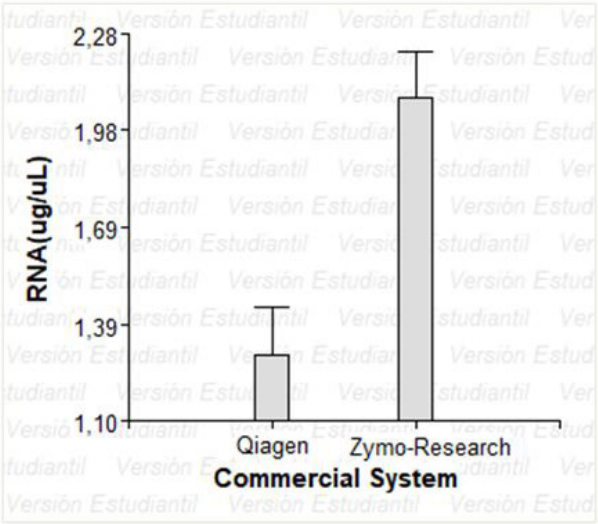


Figure 4: The Zymo research system using tri-reagent as a lysis buffer generated the highest values of RNA concentration, with a significant difference over the qiagen system. Difference of means: 0.78 (95%CI: 0.37-1.20); p-valor: 0.00054

To detect differences between the means of the variables absorbance 260/280 and absorbance 260/230, the nonparametric Mann Whitney U test was used, since both variables showed lack of normality, lack of equality of variances, as well as presence outliers. Evaluation and comparison of the purity of the RNA product between the two commercial systems showed that the Qiagen system was better at obtaining a product with less protein contamination, whereas the Zymo Research system was better at obtaining an RNA product less contaminated with phenols and carbohydrates, both with statistically significant differences (Table 2) (Figure 5).

Variable	U	p(2tails)
Abs(260/280)	206	0,0003
Abs(260/230)	393,5	0,0117

Table 2: Mann Withney U Test for both absorbance ratios

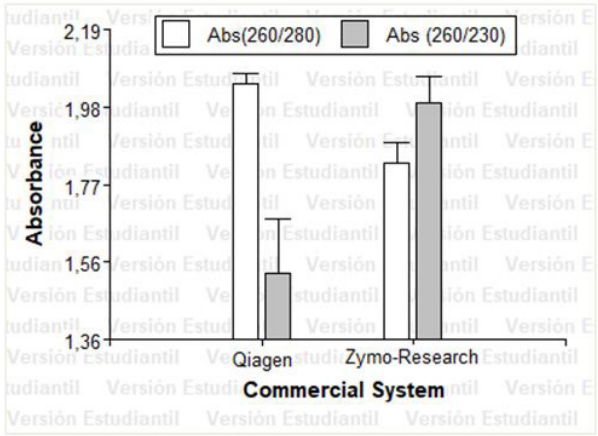


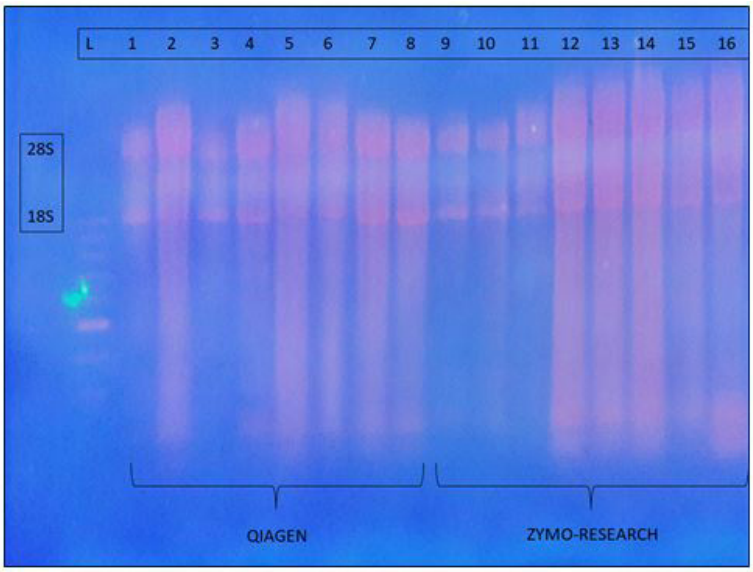
Figure 5: Average and standard error for absorbance ratios 260/280 and 260/230 of both commercial systems

No similar paper was found in scientific databases with which to contrast these results. However, studies on filamentous fungi report that the Qiagen RNeasy mini kit system is less effective for obtaining RNA extracts with optimal 260/230 ratios. For example, Sandoval *et al.* obtained RNA with low 260/230 absorbance values (<1.8) from extracts of the fungus *Xylaria* sp. using the Qiagen RNeasy mini kit [25]. This may be due to the absence of phenol in the RLT lysis buffer used by Qiagen, considering that Guzman *et al.* (2006) claim that one of the properties of phenol is to foster elimination of carbohydrates from samples [26]. In such regard, Dorrie *et al.* report that fungal RNA extracts often have a low A260/230 ratio due to contamination with melanin, which also absorbs light in the 200-400nm spectrum [27].

Furthermore, Sánchez *et al.* (2008) claim that there may be contamination by presence of residual sugars at the end of the extraction when extraction kits are used because the carbohydrates in the sample may establish hydrophobic interactions with the matrix which has the oligo dT groups necessary to capture RNA polyA+terminal sequences [28].

Evaluation of RNA Integrity

The Zymo-Research system generated an RNA product of poor quality, since 62.5% of the samples (5/8) showed signs of degradation (Figure 6). While with the Qiagen system, only 25% of the samples (2/8) showed degradation (Blasting appearance). Six of 8 samples presented in the electrophoretic run both good sharpness and better relationship between the intensity of the bands corresponding to the 28S and 18S subunits of the ribosomal DNA. According to Hernández A *et al.* (2013), an RNA extract shows integrity preserved in an agarose gel when the two bands corresponding to the 28S and 18S subunits demonstrate sharpness and the ratio of the intensity thereof should be equal to or greater than two when calculated by software for image processing [23]. If the samples are analyzed with bioanalyzers such as (Experion™systemusing the RNA StdSens Analysis chip, Bio-Rad, UK), a quality indicator of the RNA can be obtained, which ranges from 0 to 10. A value > 6.5 corresponds to extracts of optimum integrity [19].



Note: L: Ladder DNA; Street 1 to 8: RNA extracted and purified with Qiagen system; Street 9 to 16: RNA extracted and purified with Zymo-research system; Streets 5,6,12,13,14,15,16 with degradation.

Figure 6: To evaluate the integrity of the extracted RNA, it was run on 2% agarose gel, 8 samples extracted with Qiagen system, and 8 samples extracted with Zymo-research system, chosen at random.In each street 10uL of RNA was seeded with a concentration equivalent to 5ug of RNA. Note the absence in all lanes (1-16) of genomic DNA

No similar study was found in the literature with which to contrast our results regarding integrity. But according to the information obtained in this investigation, yield and spectrophotometric values are not completely reliable parameters to define the quality of an RNA extract. Other researchers such as Sandoval *et al.* and Kasajima *et al.* agree with this suggested criterion [25,29].

Evaluation of the Precision of the Qiagen Extraction System

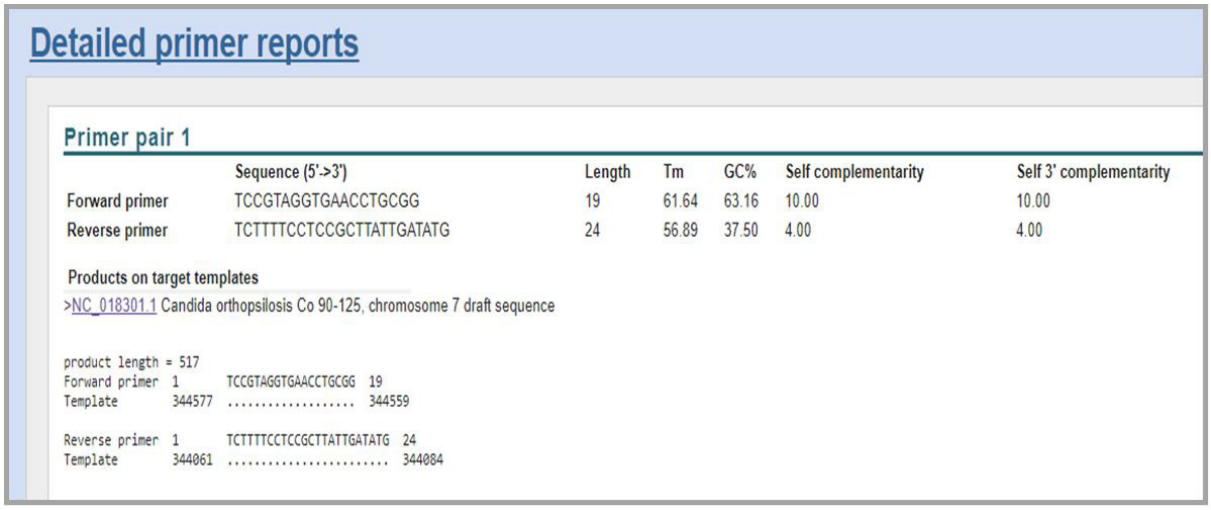
Intra-day, inter-day and inter-operator variability were measured using the proposed method. Intra-day and inter-day variability were both 9% (RSD). Intra-operator variability was 19.6% for all 3 parameters evaluated (yield, absorbance 260/280 ratio and absorbance 260/230 ratio).

Evaluation of the Precision of the Zymo Research Extraction System

Intra-day, inter-day and inter-operator variability were measured using the proposed method. Intra-day and inter-day variability were 3% (RSD) and 5% (RSD), respectively. Intra-operator variability was 22.6% for all 3 parameters evaluated (yield, absorbance 260/280 ratio and absorbance 260/230 ratio).

Reference Primer Design for the Nuclear Ribosomal DNA ITS1-ITS2 Region (Internal Transcribed Spacer)

This region was selected as a reference target because it is transcribable and present in all fungi, whether filamentous or yeast-shaped, in addition to being frequently used in phylogenetic and taxonomic studies [30]. The silico analysis of the primers demonstrated the presence of homology between the sequence of the selected primers and the ITS1-ITS2 region of the ribosomal DNA of C. orthopsilosis, which is a yeast closely related to *C. parapsilosis* sensu stricto (Table 3).



Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TCCGTAGGTGAACCTGCGG	19	61.64	63.16	10.00	10.00
Reverse primer	TCTTTTCCTCCGCTTATTGATATG	24	56.89	37.50	4.00	4.00

Products on target templates

>NC_018301.1 Candida orthopsilosis Co 90-125, chromosome 7 draft sequence

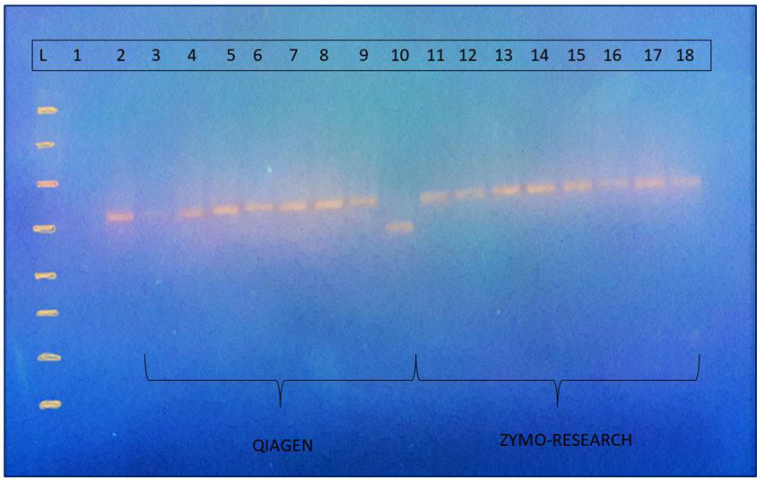
```
product length = 517
Forward primer  1       TCCGTAGGTGAACCTGCGG  19
Template        344577  ...................  344559

Reverse primer  1       TCTTTTCCTCCGCTTATTGATATG  24
Template        344061  ........................  344084
```

Table 3: Screenshot of the result thrown by PrimerBLAST about the features, sequence and specificity of the primers used for RT-PCR

Following the criteria of Sandoval-Pineda *et al.* and with the aim of predicting the efficiency of the primer before its in vitro validation in PCR reactions, the mFold software was used to determine the tendency of the primers to form secondary structures. It was found that optimal energy (ΔG) is -2.01 and 1.95 Kcal/mol for sense and antisense primers, respectively. This value refers to the minimum amount of energy required to break said structures. ΔG values lower than -9Kcal/mol may cause problems in PCR reactions, usually associated to high GC content which would requiere aggressive denaturing conditions such as higher dissociation temperatures, causing rapid deterioration of the polymerase [25].

According to Dieffenbach *et al.* a pair of primers has low probability of forming secondary structures when Tm ranges from 50 to 60 ˚C; the difference of the Tm of the two sequences should not exceed 2 ˚C; and with autocomplementary values at the 5´and 3´ ends of: (ANY<3; and 3'<6) [31]. However, the in silico analysis did not provide perfect values on primer thermodynamic characteristics, particularly for the forward primer. Nevertheless, the in vitro validation enabled us to confirm the usefulness of the pair of primers, since we were able to obtain the desired amplification product (Figure 7).



Note: Sample 10 throws a band of smaller size to the others. Probably it is a different genotype of the same species, since the strain is confirmed by sequencing.that it is *Candida parapsilosis* sensu stricto.

Figure 7: Electrophoretic run of PCR products using primers ITS1-ITS4. L=Ladder DNA 100pb; Street 1=Negative control; Street 2=Positive control, with genomic DNA (10uL/100ng) of reference strain (ATCC 22019); Street 3=Detection limit control with sample cDNA (1uL/1ug); Streets of 3 to 10=cDNA of samples processed with Qiagen ; Streets of 11 to 18=cDNA of samples processed with Zymo-Research

The ITS1 and ITS4 primers were validated and optimized by an annealing temperature gradient, where the optimum temperature was 55 ˚C, temperatures lower than 50 ˚C favored non-specific amplifications and temperatures higher than 61 ˚C inhibited primer hybridization (data not shown). A sensitivity test was used to determine that the pair of primers used has an amplification limit of up to 100ng of cDNA (Figure 7). Khalili *et al.* (2016) used the 18S gene of ribosomal DNA to normalize the expression levels of the *Hsp90* gene in *Candida albicans* isolates obtained from patients with different geographical origin; obtaining satisfactory results. This is one of the few studies that used a region of ribosomal DNA as a reference gene for a qRT-PCR study [32].

RT-PCR Result

RT-PCR provided the expected amplification product (517pb) in all samples evaluated (8 processed with Qiagen and 8 processed with Zymo-Research), and both cDNA dilutions (1:10 and 3:10) produced bands. This means that RNA purified with both systems had sufficient qualities to be amplified by PCR, despite contamination with proteins and carbohydrates and partial degradation in some samples, although the bands produced by RNA extracts obtained with Qiagen were sharper than those produced by RNA extracts obtained with the Zymo Research system. Nonetheless, rRNA band intensity ought to be even for all samples regardless of the commercial system used, since the expression of that region should be consistent among all strains, as they all belong to the same species (Figure 7).

These results suggest that the RNA obtained using the Zymo Research protocol may have reduced the efficiency of the RT-PCR reaction. One of the causes may be the composition of the lysis buffer, considering that Sánchez *et al.* (2008) report that even after centrifugation, considerable amounts of lysis buffer may remain trapped within internal spaces of amorphous precipitated matter, possibly affecting the efficiency of the PCR reaction, ultimately producing tenuous bands in the electrophoresis gel [28].

The sequences obtained, using the Sanger sequencing technique, demonstrated by NucleotideBLAST, more than 90% identity with the ITS1-ITS2 region of the ribosomal DNA of *Candida parapsilosis*; with an e-value of 0.0, which means that the homology found was not a product of random (Table 4).

Description	Max score	Total score	Query cover	E value	Ident	Accession
Candida parapsilosis strain LV2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribo	1206	2202	95%	0.0	91%	KP895597.1
Candida parapsilosis strain V3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos	1105	1590	91%	0.0	90%	KP895608.1
Candida parapsilosis strain SC1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribo	1031	1783	96%	0.0	96%	KF953899.1
Candida parapsilosis strain BKR1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S rit	996	1748	95%	0.0	95%	KC462059.1
Candida parapsilosis isolate ZA031 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, co	989	1555	95%	0.0	94%	FJ662413.1
Candida parapsilosis strain CCTCC AY93024 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence;	848	1413	94%	0.0	95%	HQ398238.1
Candida parapsilosis strain CP1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large su	839	1392	89%	0.0	99%	MF510433.1
Candida parapsilosis isolate SDMd13 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28	821	1348	92%	0.0	95%	KM265109.1
Candida metapsilosis strain EL2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribo	819	1208	87%	0.0	88%	KP895602.1
Candida orthopsilosis 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequer	819	1469	95%	0.0	89%	JQ726603.1
Candida parapsilosis strain WC58-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, co	819	1400	95%	0.0	97%	EF191046.1
Candida parapsilosis genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequences, strain IFM 52618	815	1387	94%	0.0	97%	AB109284.1
Candida parapsilosis isolate CP42 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large	811	1372	92%	0.0	97%	MF462176.1
Candida parapsilosis strain B192A internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S r	811	1377	94%	0.0	97%	KP674519.1

Table 4: Screenshot of the results obtained by the BLASTn algorithm on the identity of the PCR product obtained with the ITS1-ITS4 primers

Whatever extraction method is used to obtain nucleic acids from fungal cells, we believe it is important to highlight that fungi, like encapsulated and Gram-positive bacteria, pose a challenge for nucleic acid extraction methods. In true fungi such as yeasts and filamentous fungi, the main obstacle for any nucleic acid extraction method is undoubtedly the cell wall, which is a matrix made up of three main components: chitin (with greater presence in filamentous fungi than in yeasts), glucans and proteins [33]. Oomycetes and Myxogastria, which for a long time were considered part of the "fungus" kingdom, are currently considered pseudo-fungi because they have cell walls of similar composition, but structural and molecular evidence has reclassified them as heterokonts, related to autotrophic brown algae and diatoms. In contrast to fungi, Oomycetes typically have cell walls composed of cellulose and glucans instead of chitin [34]. It is very important to consider these cell wall features whenever an experiment is designed to extract nucleic acids from fungi or pseudo-fungi because, according to Francesconi *et al.* (2008), the different cell wall components have a significant effect on the quality of the DNA and RNA extracts, with dramatic impact on the outcomes of genetic studies [35].

Techniques for breaking down fungal cell walls include: (a) techniques based only on enzymatic methods (zymolyase or liticase), (b) techniques based on physical treatment by freezing with liquid nitrogen followed by grinding with mortar and pestle38 or shaking with beads, and (c) methods combining physical and enzymatic or physical and chemical treatments [36-41].

The cell walls of yeasts have a lower percentage of chitin, enabling the use of lest strict methods for DNA and RNA extraction [33]. Yeast DNA extraction protocols published to date basically use physical treatments with glass beads combined with an enzymatic or chemical method. However, extraction and purification techniques need to be adapted when the target is RNA, because it is unstable and more sensitive to endogenous and exogenous nuclease action (environment, operator) [40,41]. The only paper published using a yeast model to obtain RNA extracts is Mutio *et al.* (2005), which employs glass beads to break down the cell wall in presence of a buffer with guanidine to inhibit ribonucleases, complemented with acid phenol and silica columns to purify the target [42]. Said study reported excellent results for both yield and 260/280 absorbance ratio. Table 5 shows the advantages and disadvantages of the different lysis systems for RNA extracts which have been tested on yeasts and other types of fungi. Following Klassen *et*

al. (2008), we used a spheroplast-forming solution based on sorbitol, phosphate buffer, beta-mercaptoethanol and zymolyase to permeabilize the cell walls of the yeast *Candida parapsilosis* sensu stricto [43]. This choice was based on the good results obtained with zymolyase in nucleic acid extraction protocols in yeasts; and the fact that the addition of beta-mercaptoethanol to the solution potentiates the effect by protecting RNA from ribonuclease action due to its ability to eliminate disulfide bonds [36,43,44].

Lysis system	Mechanism of action	Advantages for the target and operator	Disadvantages for the target and operator
Zymolase (Querol *et al.* 1992) [36] (Klassen *et al.* 2008) [43] (Suzuki *et al.* 2013) [45]	Enzymatic: hydrolyzes glucose polymers linked by β-1,3-bonds, producing laminaripentaose.	High efficiency.. No toxicity, and avoid the use of phenol chloroform. Does not affect the integrity of RNA.	Consumption of time, high cost. According to Suzuki *et al.* enzymatic treatment can generate changes in gene expression. However this has not been validated.
Beta-Glucoronidase (Cadavid E, *et al.* 2009) [46]	Enzymatic: catalyzes the reaction Beta-D-glucuronoside+ H2O ↔D-glucuronate+ Alcohol	Fast obtaining of DNA, in good concentration and of high quality. Decrease in time and costs.	There are no experiences with RNA.
Betamercaptoethanol (Nelson *et al.* 2005) [47]	Chemical: reducing agent, reduces disulfide bridges.	Irreversibly denatures ribonucleases. Protects the RNA	It`s toxic for to the operator. Requires combining with another method
SDS: sodium duodecyl sulfate (Rojas *et al.* 2011) [48] (Rodrigues *et al.* 2017) [41]	Chemical: ionic detergent, denatures proteins.	It does not affect the integrity of the RNA. No toxicity and low cost.	Contaminates the RNA with DNA. Requires DNase purification. Inhibits PCR at minimal concentrations. Requires combining with another method.
CTAB: hexadecylmethyl-ammonium bromide (Rodrigues *et al.* 2017) [41] (Sandoval *et al.* 2017) [25]	Chemical: detergent.	Reduce contamination with carbohydrates. No toxicity. Low cost.	It does not protect the RNA from degradation. Time consuming. It requires combining with another method.
Trizol/Tri-reagent (Phenol, chloroform, more guanidine isothiocyanate) (Chomczynski 1993) [49] (Sandoval *et al.* 2017) [25]	Chemical: Denatures and removes proteins.	It is an RNA stabilizer, Inhibits RNases. Good quality and integrity of RNA extracts have been reported in Sacharomyces ceriviciae model	It is toxic, requires cabin management and protection barriers. It can generate contamination with carbohydrates. It decreases the performance of the PCR at concentrations of 0.2% and 0.5% completely inhibits it. High cost and time consuming. Requires combining with another method.
Glass beads(Hoffman y Winston, 1987) [50]	Mechanical: breaks the cell wall by hitting.	No toxicity for the operator. Low cost.	It can compromise the integrity of the RNA. Requires technique and combination with another method.
Sonication (Muller *et al.* 1998) [40]	Mechanical: ultrasonic waves to stir particles	Suitable for all cell types and easily applicable in small and large scale. Save time.	It requires optimization. It can degrade the target molecule. Requires combination with another method. High price.

Table 5: Mechanism of action, advantages and disadvantages of the lysis systems most commonly used in fungi

The protocols developed and compared in this study offer both advantages and disadvantages, even though the Qiagen system has shown superiority in quality and integrity variables. The main difference between the two protocols is the lysis buffer composition, which in the Zymo-research system is a combination of acid phenol and guanidine, while Qiagen uses a lysis buffer without phenol and contains high concentrations of guanidine, providing an advantage from the standpoint of toxicity. Table 6 shows the main differences between the two extraction systems.

	Qiagen System (RNeasy Mini Kit)	Zymo-research system(Direct-zol™RNA MiniPrep)
Inoculum size	1-2x10^7	1-2x10^7
Protein denaturation and inhibition of RNasas	Isotiocianato of guanidina	Isotiocianato of guanidine + Phenol
Elimination of carbohydrates	It is unknown	Phenol
Precipitation of RNA	Column	Column
Toxicity	Low	High
Extracted material	Total RNA	Total RNA
Prize in the Argentina for 50 columns	900 USD	500 USD

Table 6: Differences between Qiagen and Zymo-research system for RNA extraction and purification

Font: Hernández A *et al*, (2013) [23]

It is important to highlight the major progress achieved in recent years regarding nucleic acid extraction and purification. From 2009 to 2011, the first papers were published reporting satisfactory results with the fluid/paper technology, showing that it is feasible to purify DNA based on filter paper chips [51,52]. In 2015 Rodriguez N *et al.* published the first paper describing an improvement in the technique, using extraction, amplification and visual detection in addition to the paper/fluid technique for

RNA. The study was designed with the aim of diagnosing Influenza H1N1 virus directly from clinical specimens. El It used an alternative Qiagen extraction method for extracting viral RNA (QIAamp Viral RNA Mini Kit) to contrast RNA yields, finding good correlation between quantities of RNA recovered by both methods. Detection sensitivity was lower in the paper-based RNA extraction method than in the standard qRT-PCR method, with a detection limit of 10^{6} copies /mL for the former and 10^{3}copies/mL for the latter [47]. In 2017 a technical variation in the system was published. It applied nanotechnology to develop a chip that uses the paper/fluid technique to extract, amplify and detect optically (UV-LED light) the direct presence of miRNAs in animal cells, with the aim of enabling early diagnosis of cancers in which these small RNAs behave as biomarkers. The results were comparable to those using the qRT-PCR reference technique [53]. Nevertheless, it should be noted that both of these papers use poor statistics to demonstrate their results.

To date, nothing has been published using the paper/fluid technology in the field of mycology. Although this new technology is time-saving and does not require centrifuges for the nucleic acid extraction step, it offers no benefit regarding toxicity, expense or the quantity of reagents and solutions needed, since it requires a lysis buffer to break down membranes, coprecipitants such as Glycoblue to increase pellet visibility, and toxic reagents such as chloroform and isopropyl alcohol, as well as the reagents required for the amplification and detection steps. We therefore consider that although paper/fluid is a promising innovative technique to speed up microbial and oncological identification or diagnostic results, there are also disadvantages which limit access to it.

Conclusions

According to our results and reports in the literature, we can say that RNA extract quality does not depend explicitly on its concentration or on its spectrophotometry values, and that is essential to check quality by electrophoretic run, even though the procedure is somewhat complex. In this context, the RNeasy mini kit (Qiagen) proved to be superior to the Zymo Research system, generating a better quality RNA product with less intra-operator variability. However, we also consider that the choice of extraction method and/or protocol should be subject to various considerations such as budget, organism, and in particular, aim of the experiment. If the aim is to detect a gene with infrequent expression and low abundance, the system of choice should be the one providing the highest quality standards. In the opposite situation, it is possible to be more flexible in the choice of the method for RNA extraction and purification.

We must take into account that in fungal cells, the presence of cell wall is a key factor, since it can impact both yield and the quality of RNA extracts. The choice should be based on the biological system, as well as on the target to be recovered. A standard criterion is that no single method is 100% effective, and a combination of techniques is always needed to increase efficiency in nucleic acid extraction protocols.

Data Support

All the data on the strains used in this work can be obtained from the database of the Institute of Research in Microbiology and Parasitology (IMPaM), Faculty of Medicine, and University of Buenos Aires.

Authorship Policy

Rodríguez ML is the principal author, who executed the investigation and analyzed the data.
Autras Rosa AC, and Jewtuchowicz VM contributed to the design of the study, and to the writing of the manuscript.

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