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RNA Extraction from the Yeast Candida parapsilosis Sensu Stricto Using Two Commercial Methods Based on Purification by Silica Columns

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Abstract

Good quality 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 or in-house IVD 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 and above all, enabling culturable fungi with low abundance as well as non-culturable fungi to be identified through analysis of their genetic material. 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.

Good quality 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 may affect RNA quality and reduce the efficiency of its amplification. 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. In addition, RNA is highly labile and less stable than DNA [1].

Different methods are currently available for disruption and homogenization of tissues with liquid nitrogen, sand, beads or mycelium lyophilization for filamentous fungi [2,3]. Techniques based on enzymatic disruption of the wall by zymolyase or lyticase are the most frequently used options for yeasts [4]. 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 hexa decyltrimethyl ammonium bromide (CTAB), and increasingly frequently, commercial extraction kits [5,6]. Nevertheless, the quality and yield of the extracted RNA may vary according to the methodology applied and the biological study species [7].

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 [8,9]. 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 [10-14]. Considering this background, it is a priority to study the transcriptome of this *Candida* species and its variations in response to environmental changes.

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 or in-house IVD techniques. 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. For extraction and purification of RNA, the

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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).

Ethical statement

Because the study uses oral fungal isolates derived from patients, the present research was treated by the ethics committee of the Faculty of Odontology, University of Buenos Aires (UBA), with file number 0048223/2016, and approved under the number 012/2016 CETICAFOUBA.

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. Each strain was kept for short periods on Sabouraud agar at 4ºC, and preserved for long periods in glycerol at -70ºC.

Culture media

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

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 min. The pellet was re-suspended in 1 mL 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 micro centrifuge at 1000 x *g* for 5 min at 4ºC.

The pellet obtained from the second rinse was re-suspended in 100 uL of spheroplast-forming solution (sorbitol+EDTA+beta-mercaptoethanol+ultra-pure nuclease-free water+1.43 mg/mL zymolase) and incubated in a laboratory water bath at 37ºC for 2 h. 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 -20ºC.

RNA extraction and purification using RNeasy Mini Kit system (Qiagen) + DNase I

Manufacturer's instructions were followed (Supplementary File 1). It should be noted that this system homogenizes the initial sample (spheroplast suspension) with guanidine isothiocyanate at high concentrations.

The treatment with DNase I (Qiagen brand) is subsequent to the addition of ethanol and prior to the rinses with the respective buffers. Manufacturer's instructions were followed.

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

Extraction and purification of RNA using the Zymo-Research+DNase I system

Manufacturer's instructions were followed (Supplementary

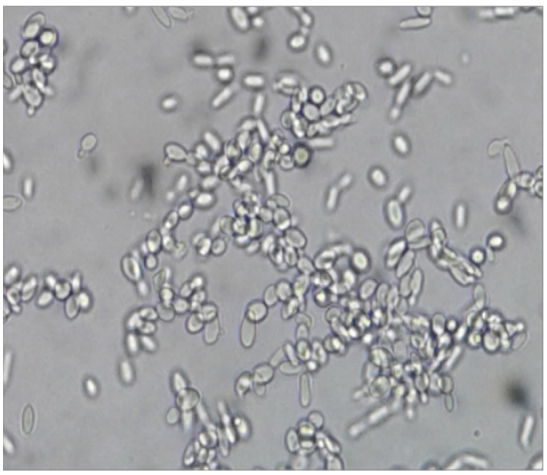


Figure 1: Yeasts with preserved cell wall, prior to the action of zymolase. (Unstained sample, 40X o.m.).

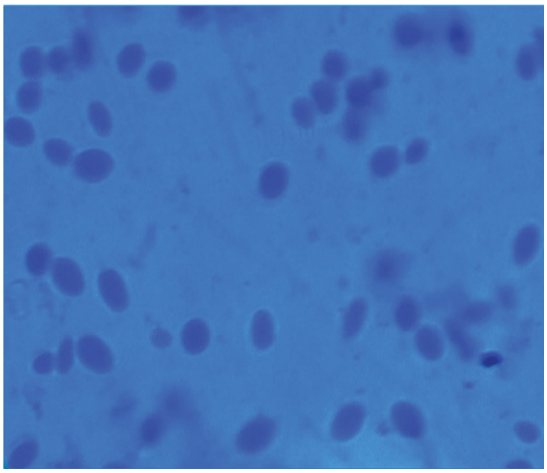


Figure 2: Yeast with altered cell wall, 2 hours after the action of zymolase. (Sample stained with toluidine blue, 40X o.m.).

File 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 in the Zymo Research kit) is after adding ethanol and prior to rinses with the respective buffers. Manufacturer's instructions were followed.

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

Analysis of yield, purity and integrity of RNA

The RNA solutions obtained were quantified using a Multiskan GO 10040/1510-02746C (Thermofisher) spectrophotometer, with wavelength 260, and the concentration in ng/µL was obtained by means of the following formula: **Abs260*40*(10/0,51)**. RNA purity was determined for each sample by means of the absorbance ratio A260/280, which measures contamination by proteins, and A260/230, which measures contamination by carbohydrates, phenols and salts.

The results for purity and yield were processed and analyzed in Microsoft Excel 2010 and InfoStat 2016 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 h and viewed in a UV trans illuminator.

RNA was preserved at -70ºC,

Preparation of copy DNA (cDNA)

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

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

The reaction was prepared in a total volume of 20 µL, constituted of 4 µL reaction mix plus 1 µL enzyme, plus nuclease-free water as required, and RNA in an amount adjustable to a concentration of 1 µg in the 20 µL reaction volume (Table 1).

Cycling conditions were the following

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 Primer Blast 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*.

The sequences obtained were the following:

ITS 1 forward: TCCGTAGGTGAACCTGCGG

ITS 4 reverse: TCTTTTCCTCCGCTTATTGATATG

The primers were validated *in silico* and experimentally. The PrimerBlast algorithm enabled primer design and showed specificity with the ITS1-ITS2 region of ribosomal DNA corresponding to Candida orthopsilosis (Figure 4), which is transcribed without modifications.

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. The aim of the RT-PCR is to obtain amplicons of the expected size (517pb). Sanger sequencing of the PCR product plus bioinformatic analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to find specificity, i.e., homology with region ITS 1-ITS2 of ribosomal DNA.

Conditions and performance of the RT-PCR

To do the RT-PCR, the RNA obtained was transcribed to cDNA (copy DNA) with the BioRad kit (iScript cDNA Synthesis kit 100 x 20 µL reactions), according to the manufacturer's instructions, based on 1 µg 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 (Figure 5).

Table 2 shows concentration and volume for each component in the RT-PCR reaction.

The PCR cycles were carried out in the Mini CyclerTM thermocycler, MJ Research INC, with the following protocol:

Statistic 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 2017. For the descriptive analysis of the variables, the average, standard deviation, median, first and third quartiles statistics were used; in addition to bar chart with standard error, and dot density chart for the comparative study.

The normality of the data was established by the Q-Q-plot test and the Shapiro Wilks test. On the other hand, the homogeneity of

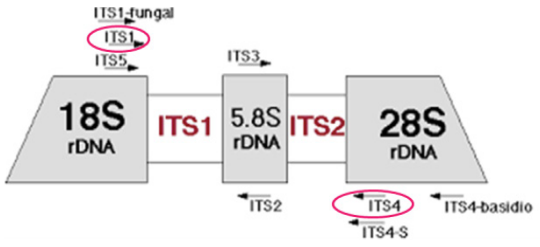
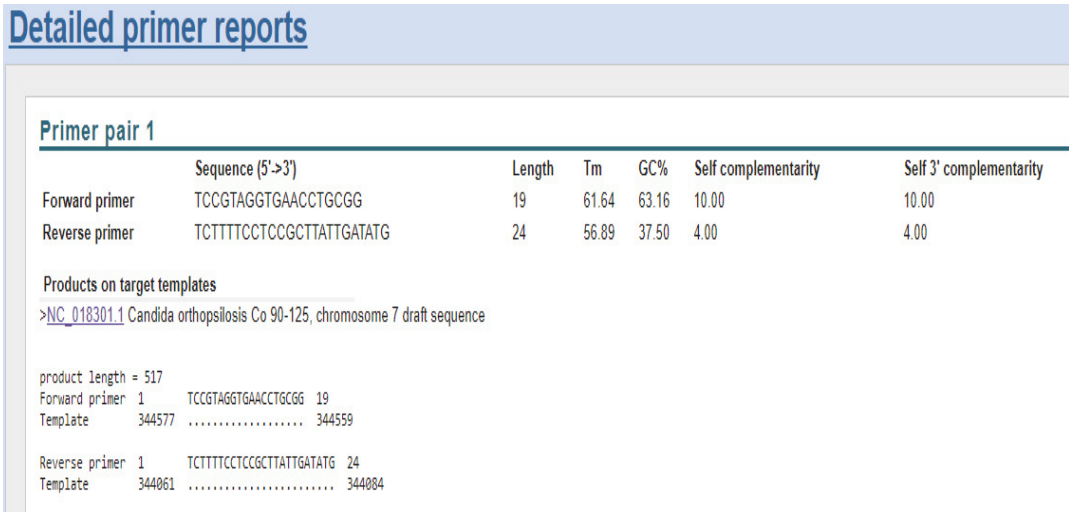


Figure 3: The ITS1 and ITS4 primers allow to raise a region of the fungal ribosomal RNA of 517bp.

Reaction protocol Incubate the complete reaction mix in a thermal cycler using the following protocol:	
Priming	5 min at 25ºC
Reverse transcription	20 min at 46ºC
RT inactivation	1 min at 95ºC
Optional step	Hold at 4ºC

Table 1: Thermocycling conditions for retrotranscription protocol.



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
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Figure 4: Characteristics, sequence and specificity of the primers used for RT-PCR.

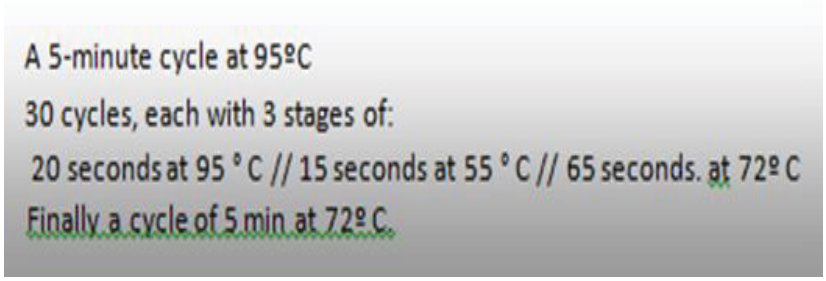

Figure 5: Amplification conditions with ITS1-ITS4 primers.

Components	Stock concentration	Final concentration	Final volume (µl)
Water			**9.85**
PCR buffer	10 X	1 X	**2.5**
Cl_2Mg	50 mM	3 mM	**1.5**
dNTPs	10 mM	0.2 mM	**0.5**
Primer ITS1	50 µM	0.4 µM	**0.2**
Primer ITS 4	50 µM	0.4 µM	**0.2**
Taq-polimerase	5 U/µl	1.25	**0.25**
cADN	**10 ng/µl**		**10 µl**

Table 2: Protocolo de PCR para amplificación de la región ITS: cantidades y concentraciones de reactivos para un volumen de 25 ul.

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) were used for independent variables; considering a value of p less than alpha error, assuming as alpha a value equal to 5%.

Evaluation of repeatability and reproducibility

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. 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 6 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. 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 [15]. To date, no study on the subject or comparing two commercial systems has been published specifically for yeasts.

To detect differences between the means of the variables absorbance 260/280 and absorbance 260/230, the nonparametric test U de Mann de Whitney 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 (Figure 7), both with statistically significant differences (Absorbance 260/280:p= 0,0003/ Absorbance 260/230:p=0,0117).

No similar paper was found in scientific databases with which to contrast these results. However, studies on filamentous fungi report that the Qiagen RN easy 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 [16]. This may be due to the absence of phenol in the RLT lysis buffer used by Qiagen, considering that Guzman et al. claim that one of the properties of phenol is to foster elimination of carbohydrates from samples [17]. 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 200-400 nm spectrum [17].

Furthermore, Sánchez et al. 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 [18].

Evaluation of RNA integrity

The Zymo Research system provided lower-quality RNA, with 62.5% of the samples (5 samples out of 8) moderately degraded (Figure 8). The Qiagen system produced RNA with better integrity, with only 25% of the samples (2 out of 8) partially degraded (Figure 8). This is consistent with absorbance at 260/280, which was significantly higher in the Qiagen system than in the Zymo-Research system.

No study was found in the literature with which to contrast our results regarding integrity. But according to the information obtained in the present study, yield and spectrophotometric values are not completely reliable parameters to define whether an RNA extract is good enough not to affect RT-PCR efficiency. Other researchers such as Sandoval et al. and Kasajima et al. agree with this suggested criterion [15,19].

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 or housekeeping 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 [20].

Following the criteria of Sandoval-Pineda et al., and with the aim

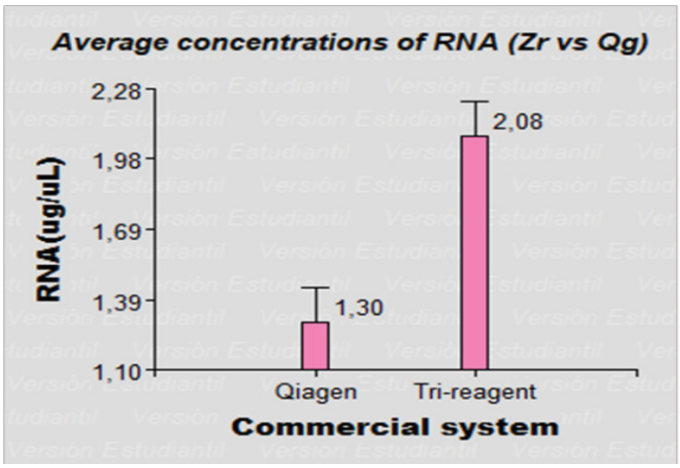

Figure 6: The Zymo research system using tri-reagent as a lysis buffer generated the highest values of RNA concentration, with a significant difference over the qiagen system.

Difference of means: 0.78/IC95%: 0.37-1.20/p-valor: 0.00054.

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 [15].

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) [21]. 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 9).

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 100 ng of DNA (Figure 9). No study which would serve for discussion of this point was identified in the literature review, since most papers on gene expression use the beta-tubulin gene as reference gene.

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 (Figure 9), 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 bought 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.

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. 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 [18-21].

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 [22]. 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 [23]. 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., 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 [24].

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 pestle or shaking with beads, and (c) methods combining physical and enzymatic or physical and chemical treatments [25-30].

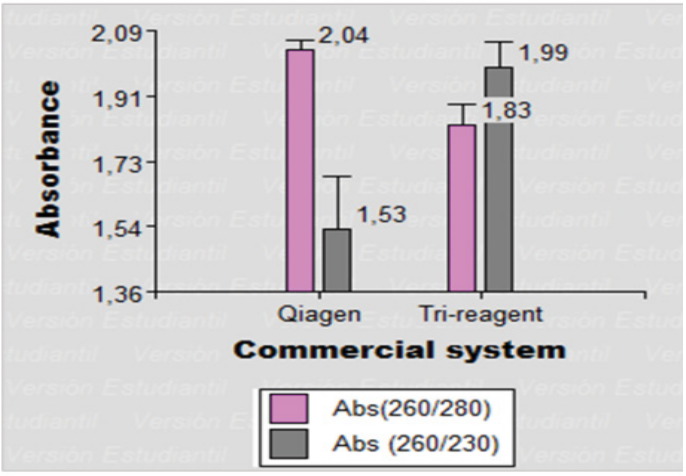

Figure 7: Average absorbance ratios 260/280 and 260/230 of both commercial systems.

Note: Qiagen: IC95% of Absorbance (260/280): 1.99- 2.10/IC95% of Absorbance (260-230): 1.23-1.84 **Zymo-research:** IC95% of Absorbance (260/280): 1,71-1,95/IC95% of Absorbance(260/230): 1.83-2.1

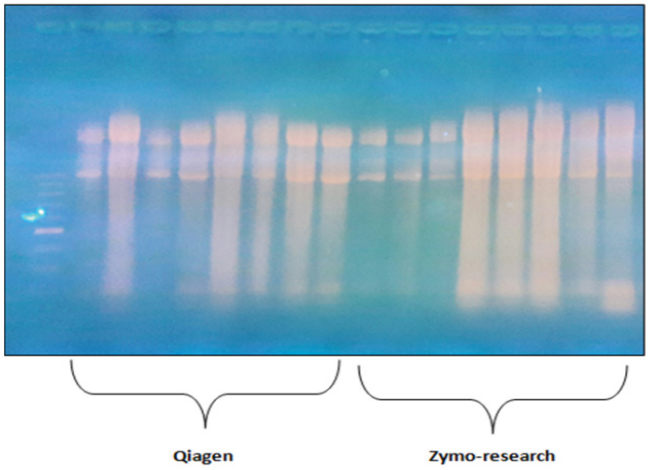

Figure 8: To evaluate the integrity of the extracted RNA, it was run on 2% agarose gel, 8 samples extracted with Qiagen system, and 8 samples extracted with Zymo-research system, chosen at random.In each street 10uL of RNA was seeded with a concentration equivalent to 5ug of RNA. Note the absence in all lanes (2-17) of genomic DNA.

Calle 1: Ladder DNA
Street 2 to 9: RNA extracted and purified with Qiagen system
Calle 10 to 17: RNA extracted and purified with Zymo-research system
Streets 6,7,12,13,14,15,16 with partial degradation.

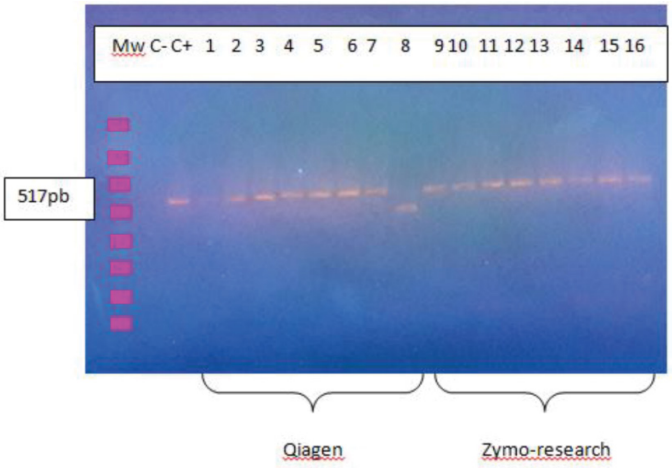

Figure 9: Electrophoretic run of PCR products:

WM= Ladder DNA 100pb. (The "ladder is over-drawn by low resolution of the reagent, because it was overdue).

C-= negative control;

C+= positive control, with genomic DNA (10uL/100ng) of reference strain (ATCC 22019)

1= detection limit control with sample cDNA (1uL/1ug) 7334;

2-8= cDNA of samples: 12A; 6912; 7066; 6PA; 6943; 75CA; 7.2.

9-16= cDNA of samples: 5301; 46A; 16A; 53A; 11.1; 5462; 14.2; 15.1

Note: Sample 8 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.

The cell walls of yeasts have a lower percentage of chitin, enabling the use of lest strict methods for DNA and RNA extraction [22]. Yeast DNA extraction protocols published to date basically use physical treatments with glass beads combined with an enzymatic or chemical method [29,30]. 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). The only paper published using a yeast model to obtain RNA extracts is Mutio et al., 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 [31]. Said study reported excellent results for both yield and 260/280 absorbance ratio. Table 3 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., we used a spheroplast-forming solution based on sorbitol, phosphate buffer,

Lysis system	Mechanism of action	Advantages for the target and operator	Disadvantages for the target and operator
Zymolase Querol et al. [25] Klassen et al. [32] Suzuki et al. [43]	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. [42]	Enzymatic: Catalyzes the reaction Beta-D-glucuronoside + H_2O ↔ 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. [33]	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. [34] Rodrigues et al. [30]	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: Hexadecylmethylammonium bromide Rodrigues et al. [30] Sandoval et al. [15]	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 [35] Sandoval et al. [15]	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 [36]	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. [29]	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 3: Mechanism of action, advantages and disadvantages of the lysis systems most commonly used in fungi.

	Qiagen System (RNeasy Mini Kit)	Zymo-research system (Direct-zol™RNA MiniPrep)
Inoculum size	1-2 x 10^7	1-2 x 10^7
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

Font: Hernández A et al. [37]

Table 4: Differences between Qiagen and Zymo-research system for RNA extraction and purification.

beta-mercaptoethanol and zymolyase to permeabilize the cell walls of the yeast *Candida parapsilosis* sensu stricto [32]. 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 [25,32,33].

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 3 shows the main differences between the two extraction systems [34-37].

Los protocolos desarrollados y comparados en este estudio ofrecen tanto ventajas como desventajas, más allá de que el sistema de Qiagen haya demostrado superioridad en las variables de calidad e integridad. La principal diferencia en ambos protocolos se centra en la composición del buffer de lisis, siendo éste una combinación de fenol ácido y guanidina para el caso del sistema Zymo-research. Mientras que Qiagen emplea un buffer de lisis que carece de fenol y que contiene guanidina en altas concentraciones, lo cual es una ventaja desde el punto de vista de la toxicidad. En la tabla 6 se exponen las principales diferencias entre los dos sistemas de extracción (Table 4).

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 [38,39]. In 2016, Rodriguez 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 [40]. 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 [41-43]. 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.

Conclusion

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 Qiagen RNeasy mini kit 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. It should be borne in mind 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.

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