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Evaluation of methods for the detection of low-abundant snoRNA-derived small RNAs in *Saccharomyces cerevisiae*

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Abstract

In recent years, there are a growing number of studies demonstrating the existence of small RNAs derived from snoRNAs (sdRNAs) in multiple eukaryotic organisms. Such RNAs have been initially observed in high throughput sequencing studies and assumed to be processed by miRNA machinery. Recently, we have identified sdRNAs that are associated with ribosomes in yeast *Saccharomyces cerevisiae*. Although sdRNAs were detectable in sequencing data, their low abundance hampered their detection by other methods. Here, we present the results of our survey for optimized experimental method for sdRNA detection. We have compared two extraction procedures of total RNA from *S. cerevisiae*: MasterPure™ kit and Trizol with two methods resulting in enrichment in small RNA fraction and MasterPure™ with selective isopropanol precipitation and bulk tRNA isolation methods. Also the sensitivity of three methods for sdRNA detection was verified: a northern blot using standard or LNA probes and stem-loop reverse transcription followed by PCR (SL-RT-PCR). Our results reveal that Trizol isolation method combined with SL-RT-PCR is the most effective in the detection of low-abundant sdRNAs.

Key words: snoRNA, sdRNA, small RNA, ribosome-associated RNA, rancRNA, *Saccharomyces cerevisiae*

Introduction

Small nucleolar RNAs (snoRNAs) are highly evolutionarily conserved class of RNAs, which are present throughout the eukaryotes, and are classified into two groups, namely, C/D and H/ACA box that function as ribonucleoprotein (RNP) complexes to guide enzymatic modification of the target RNAs at sites determined by RNA : RNA antisense interactions. Generally, most of C/D box snoRNAs are 70-120 nucleotides (nt) long guiding methylation of the target RNAs, while H/ACA box snoRNAs are usually 100-300 nt long guiding pseudouridylation. These RNAs were initially discovered in nucleolus and thought to exclusively target ribosomal RNAs inside this sub-nuclear compartment. However, the finding that numerous snoRNAs do not possess target RNAs (Huttenhofer et al. 2001; Jady and Kiss 2000; Cavaille et al. 2000; Vitali et al. 2003) opened us (scientists) to new possibilities concerning snoRNAs' functions and targets. For example, SNORD114-1 snoRNA has been shown to promote G0/G1 to S phase transition through cell cycle and to be deregulated in cancer cells (Valleron et al. 2012). Another C/D box snoRNA, SNORD115 (HBII-52), reveals the sequence complementarity to the alternative splice site of serotonin receptor 2C pre-mRNA, and thus influences its alternative splicing (Kishore and Stamm 2006). Unexpectedly, some canonical snoRNAs with known ribosomal targets (SNORD32A (U32A), SNORD33 (U33), and SNORD35A (U35A)) have been shown to accumulate in the cytosol under cellular stress conditions in higher eukaryotes (Michel et al. 2011). Similar to other RNA species, they can be subjected to degradation and processing performed by non-nuclear RNases. Recently, several reports have identified small (18-22 nt) RNA derivatives of snoRNAs, termed as "snoRNA-derived RNAs" (sdRNAs, reviewed in Falaleeva and Stamm 2013 and Tyczewska et al. 2016). It has been shown that several sdRNAs reveal miRNA-like properties, or regulate alternative mRNA splicing (Ender et al. 2008; Brameier et al. 2011; Kishore et al. 2010). Parallel next-generation sequencing

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studies of the small transcriptome in mice revealed the presence of snoRNA-originating miRNAs in embryonic stem cells and demonstrated that such sdRNAs exhibit tissue-specific expression profiles (Babiarz et al. 2008; Babiarz et al. 2011). At the same time, snoRNA-originating miRNA-like molecules were described in the protozoan *Giardia lamblia*, a unicellular parasite whose genome does neither encode Drosha nor Dicer, suggesting the existence of an alternative processing pathway (Saraiya and Wang 2008; Li et al. 2011). One year later, the same snoRNA-processing event was described in cells infected with Epstein-Bar virus (EBV). In this system, a miRNA-like precursor endogenously encoded by a viral v-snoRNA1 is expressed upon induction of the lytic cycle to suppress viral DNA polymerase (Hutzinger et al. 2009). miRNA-independent pathway of sdRNA maturation and function could also be expected in the budding yeast *Saccharomyces cerevisiae*, which does not possess components of the machinery necessary for microRNA action (Houseley and Tollervey 2008). Indeed, in our previous study, 10 known sdRNAs from *S. cerevisiae* have been identified as ribosome-associated ncRNAs (rancRNAs) (Zywicki et al. 2012). These yeast snoRNA fragments possess different length than typical microRNA-like sdRNAs, ranging from 18 nt to 60 nt. Although sdRNAs were observed in very low abundance within our cDNA libraries, their regulatory potential cannot be excluded. Our recent studies demonstrated that even a relatively small amount of 18-mer rancRNA (~27 000 molecules/cell) is sufficient to substantially influence global ribosome activity (~200 000 ribosomes/cell) and switch translation in the cell (Pircher et al. 2014).

Emerging novel regulatory potential of sdRNAs demonstrate that reliable detection of sdRNA expression is essential for better understanding of sdRNA-mediated gene expression regulation. However, due to their low abundance, conventional techniques such as cloning, northern hybridization, and microarray analyzes may not be sensitive enough to detect the complete repertoire of sdRNAs. This has been proven for low-abundant miRNAs that routinely escape detection with cloning, northern hybridization (Lim et al. 2003), and microarray analyzes (Krichevsky et al. 2003). Using the sensitive reverse transcription-polymerase chain reaction (RT-PCR) detection method, poor sensitivity and low throughput of conventional technologies can be overcome. However, the detection of sdRNAs by PCR is technically demanding due to their small size. A number of specific RT-PCR techniques were developed and optimized for miRNA detection, including real-time methods based on reverse transcription (RT) reaction with a stem-loop primer followed by a TaqMan PCR analysis (Chen et al. 2005; Tang et al. 2006; Varkonyi-Gasic et al. 2007). The stem-loop reverse transcription primers ensure higher specificity and sensitivity than linear primers because of base stacking and spatial constraints of the stem-loop structure (Chen et al. 2005). Detection sensitivity can be further increased by a pulsed RT reaction (Tang et al. 2006). These methods were, however, optimized for the detection of miRNAs, which are supposedly more abundant than sdRNAs.

On the contrary, it has also been shown that different purification methods may significantly affect the composition of RNA species in isolated RNA fractions (Kim et al. 2012). Several studies have tackled this point, focusing on methods for miRNA extraction (Monleau et al. 2014; Li and Kowdley 2012; Podolska et al. 2011); however, similar considerations could also be true in analyzing sdRNAs.

Here, the suitability of the northern blot and stem-loop RT-PCR (SL-RT-PCR) methods in detecting small RNAs derived from snoRNAs in *S. cerevisiae* was compared. Different RNA isolation methods that could vary in recovery of small RNA fraction were also evaluated. We provide evidence that low-abundant *S. cerevisiae* sdRNAs can be easily detected with SL-RT-PCR method with as little as 50 ng of low-molecular-weight RNA (LMW RNA, 10-60 nt). Using such protocol, we were able to robustly detect the expression of three sdRNAs identified in our previous studies as ribosome-associated RNAs (Zywicki et al. 2012).

Materials and methods

Strains and growth conditions

S. cerevisiae wild-type strain BY4741 (MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) was grown in YPD medium at 30 °C.

RNA isolation

Using the following two different methods, total RNA was isolated from *S. cerevisiae*: 1) MasterPure™ Yeast RNA Purification kit (Epicenter) and 2) Trizol (Ambion), according to manufacturer's protocol.

Table 1. The sdRNAs, primers, and probes used in this study

Name	Type	Sequence [5′-3′]	Length [nt]
5′-sdRNA 67	**sdRNA**	**AACAUGAUGACUAAGUUGU**	**19**
	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAACT	50
	Fwd primer	GGCGCGCGCGAACATGATGACTA	23
sdRNA 83	**sdRNA**	**GGACCAAUUACCGUAGUUGCGACUACAACAAUUUUGUUCAUA**	**42**
	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTATGAA	50
	Fwd primer	GACCAAUUACCGUAGUUGCGAC	22
	NB LNA	TATGA*ACA*CAATTG*TTGTA*GT	21
5′-sdRNA 128	**sdRNA**	**CACGGUGAUGAAAGACUGGUU**	**21**
	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACCAG	50
	Fwd primer	GCGCGGGCACGGTGATGAAAGA	22
	NB aDNA	ACCAGTCTTTCATCACCGTGA	21
3′-sdRNA 128	**sdRNA**	**UCCUAGGAUGUCUGAGUG**	**18**
	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACTCA	50
	Fwd primer	CGGCGGCCTCCTAGGATGTC	20
3′-sdRNA 4	**sdRNA**	**CCUUUAUAGCGGUGCUUUAACUAUUAAUAACU**	**32**
	NB aDNA	AGTTATTAATAGTTAAAGCACCGCTATAAAGG	32
	Uni primer	CCAGTGCAGGGTCCGAGGTA	20

Abbreviations: RT primer – stem-loop primer used for reverse transcription; Fwd primer – forward PCR primer; NB LNA – LNA probe for northern blot hybridization; NB aDNA – DNA probe for northern blot hybridization; Uni primer – universal PCR primer; sdRNA sequences are presented in bold. Position of LNA modification is underlined and marked with *

The RNA enriched in small RNA fraction (up to ~120 nt) was isolated according to two different protocols. First, MasterPure™ Yeast RNA purification kit (Epicenter) was combined with the enrichment of LMW RNAs with isopropanol. Briefly, RNA fraction longer than ~120 nt in size was precipitated using one third volume of isopropanol and discarded. Next, small RNAs (up to ~120 nt) that remained in the supernatant were precipitated using one volume of isopropanol. Second, bulk (unfractionated) tRNAs from *S. cerevisiae* were prepared as previously described (Monier et al. 1960). Briefly, unbuffered phenol 90% (equilibrated with water, Sigma) was added to cell lysates and mildly shaken at room temperature. Under such mild phenol treatment, preferentially the "soluble" RNAs (essentially tRNA, 5S-RNA, and small cellular RNAs) are released from the unbroken cells (Monier et al. 1960). The possible contamination of large ribosomal RNA was removed with 2 M LiCl.

For SL-RT-PCR method, Trizol-isolated total RNA was loaded on 10% denaturing polyacrylamide gel and LMW RNAs (LMW, 10-60 nt) were eluted from gel. Briefly, the bands of interest were excised from the gel with a razor blade. The gel slice was crushed, soaked in the elution buffer (300 mM NaOAc, 1 mM EDTA), and incubated with shaking for at least 16 h at 4°C. LMW RNAs were recovered from the eluate by ethanol precipitation. The concentration of RNA was determined using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

Northern blot analysis

Of about 50-100 μg of RNAs were separated on 12% denaturing polyacrylamide gels and electrotransferred (45 min, 0.8 mA/cm^2 of a membrane) to positively charged Amersham Hybond N$^+$ membrane using a semi-dry blotter (BioRad). Nucleic acids were UV-cross-linked to membranes, which were then used immediately for northern blot hybridization or stored at room temperature. DNA oligonucleotide probes were synthesized by Genomed, LNA probe by Future Synthesis (Table 1). Hybridization was carried out overnight in 30 ml of a buffer (178 mM Na_2HPO_4, 882 mM NaH_2PO_4, 7% SDS). Two-

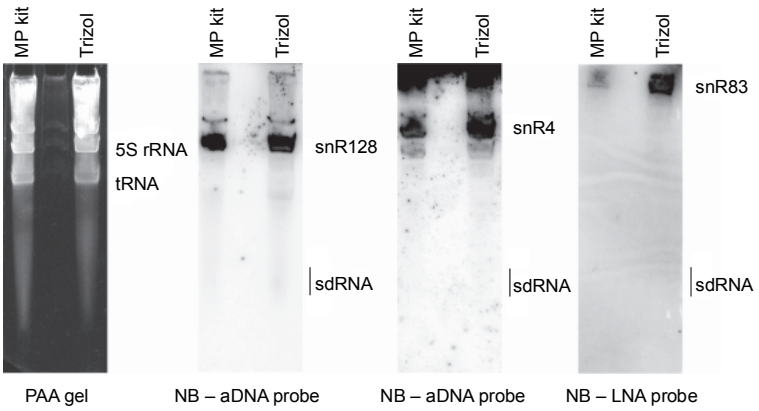

Fig. 1. Northern blot hybridization membranes with the total RNA. Denaturing polyacrylamide gel electrophoresis (left) and northern blot hybridization results (right) with 100 μg of total RNA isolated with MasterPure™ kit (MP kit, lane 1) and Trizol (lane 2). Position of tRNAs and 5S rRNA are marked on gel. Positions of full-length snoRNAs and estimated position of sdRNAs are marked on hybridization membranes

step washing was performed after hybridization: for 2 min in a washing solution I (2 × SSC, 0.1% SDS) and for 1 min in a washing solution II (0.1 × SSC, 0.1% SDS). Membranes were exposed overnight on the phosphor – storage intensity screen (Fujifilm). Screens were scanned with Fujifilm Fluorescent Image Analyzer FLA-5100. Size estimates for detected RNAs were determined using RNA markers (Promega or Invitrogen).

Stem-loop pulsed reverse transcription combined with PCR (SL-RT-PCR)

Reverse transcription reactions contained 10–200 ng of RNA samples, 50 nM stem-loop RT primer, 1 × RT buffer, 0.25 mM of each dNTPs, 50 U SuperScript SSIII reverse transcriptase (Invitrogen), 5 U RiboLock RNase Inhibitor (Thermo Scientific), and 10 mM of DTT. About 20 μl reactions were incubated in a Bio-Rad T100™ Thermocycler for 30 min at 16 °C, followed by pulsed RT of 60 cycles at 30 °C for 30 s, 42 °C for 30 s, and 50 °C for 1 s. To inactivate the reverse transcriptase, samples were incubated at 85 °C for 5 min and then held at 4 °C. All reverse transcriptase reactions, including no template controls, were run in triplicate. About 50 μl PCR reaction included 2 μl RT product, 25 μl of 2 × DreamTaq MasterMix, and 0.2 μM primers. The reactions were incubated at 95 °C for 3 min, followed by 25-31 cycles of 94 °C for 30 s, and 60 °C for 30 s, followed by 72 °C for 30 s. All reactions were run in triplicate.

The stem-loop RT primers were designed according to Chen et al. (2005). The specificity of SL-RT primers to individual sdRNA was conferred by a six nucleotide extension at the 3′ end; this extension was a reverse complement of the last six nucleotides at the 3′ end of the sdRNA (Table 1). Forward primers were specific to sdRNA sequence but excluded the last six nucleotides at the 3′ end of sdRNA. A 5′ extension of 5-7 nucleotides was added to each forward primer to increase melting temperature of the primers; these sequences were chosen randomly and are relatively GC-rich. We used Primer3Plus design software to assess the quality of forward primers.

Results

Hybridization-based technologies fail to detect sdRNAs

At the very first step, we decided to compare the efficiency of recovery of snoRNA fragments between two different methods of total RNA isolation: MasterPure™ Yeast RNA Purification kit and Trizol. After loading 100 μg of total RNA on 12% polyacrylamide gels and SYBR® Safe staining, we clearly observed good separation of distinct RNAs, including bulk tRNAs, 5S rRNA, and a portion of small RNAs (Fig. 1, left panel). Northern blot experiments were performed using antisense DNA (aDNA) probes specific for sdRNAs derived from snR128 and snR4 and LNA probe specific for sdRNA derived from snR83 (Table 1). In all tested cases, clear signals derived from full-length snoRNAs were observed (Fig. 1). However, both of the total RNA isolation methods failed to provide amounts of sdRNA fragments

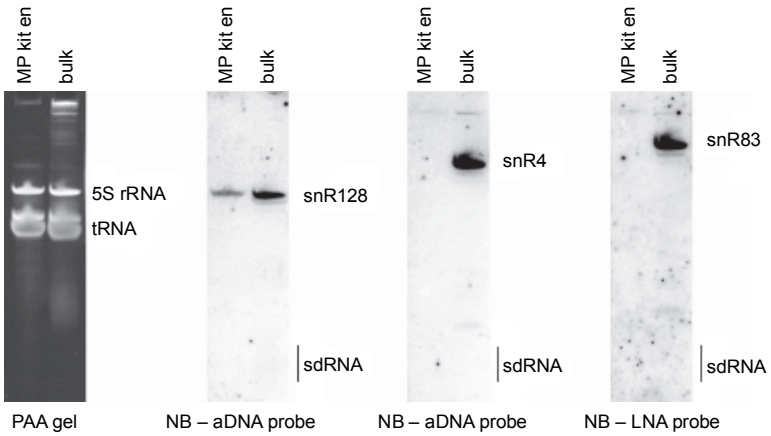

Fig. 2. Northern blot hybridization membranes with the total RNA enriched in small RNA fraction. Denaturing polyacrylamide gel electrophoresis (left) and northern blot hybridization results (right) with 25 μg of small RNAs (up to ~120 nt) isolated with MasterPure™ kit followed by selective small RNA precipitation (MP kit en, lane 1) and the bulk RNA isolation method (lane 2). Position of tRNAs and 5S rRNA are marked on the gel. Positions of full-length snoRNAs and the estimated position of sdRNAs are marked on hybridization membranes

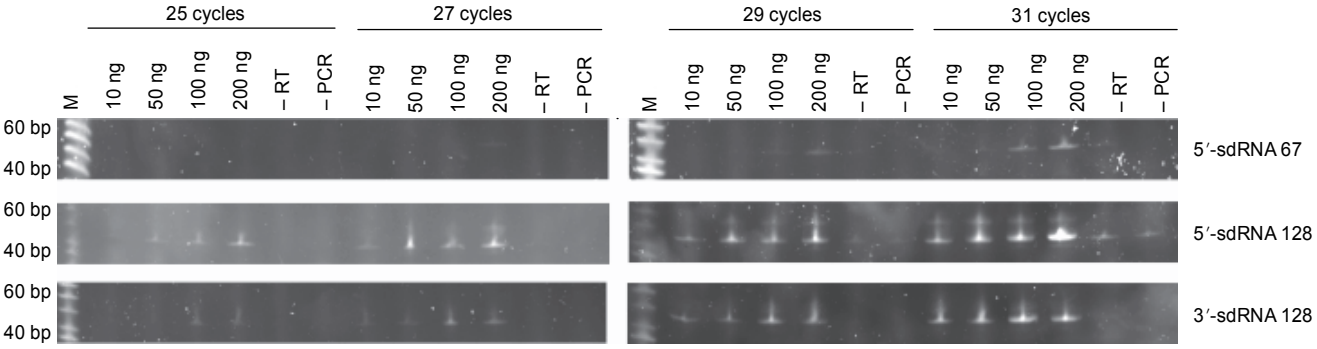

Fig. 3. Stem-loop RT-PCR assay for sdRNAs. Stem-loop RT-PCR analyses of the expression of sdRNAs: 5′-sdRNA 67, 5′-sdRNA 128 and 3′-sdRNA 128. About 10-200 ng of low molecular weight RNA (10-60 nt) was used for reverse transcription reactions. The number of PCR cycles is indicated at the top of lanes

above the clear detection threshold with either aDNA or LNA probe (Fig. 1).

Therefore, in order to maximize and concentrate the sdRNAs' amount within the tested RNA pools, total RNA fractions were enriched with small RNA fractions, up to ~120 nt in size. We have 1) combined MasterPure™ Yeast Purification kit with the selective isopropanol precipitation of small RNAs and 2) used bulk tRNA isolation method that results in isolation of soluble RNAs, including 5S rRNA, tRNAs, and small RNAs (Monier et al. 1960). After loading 25 μg of small RNAs on 12% denaturing polyacrylamide gels and SYBR® Safe staining, we observed a good separation of distinct RNAs, including bulk tRNAs, 5S rRNA, and a portion of small RNAs (Fig. 2, left panel). Northern blot experiments were performed using antisense DNA (aDNA) probes specific for sdRNAs derived from snR128 and snR4 and LNA probe specific for sdRNA derived from snR83 (Table 1). We have observed clear signals derived from full-length snoRNAs within the small RNAs isolated with the bulk method (Fig. 2). We have observed that MasterPure™ Yeast Purification kit with the selective isopropanol precipitation of small RNAs' method resulted in purification of RNAs of ~120 nt and less. In this case, northern blot signals were observed from full-length snR128 (126 nt in length) but longer snoRNAs: snr4 (186 nt) and snR83 (306 nt) were not detected. Moreover, we did not observe any significant improvement in terms of

sdRNA detection since both of the small RNA-enrichment methods failed to provide amounts of sdRNA fragments above the clear detection threshold either with aDNA or LNA probe (Fig. 2).

Stem-loop RT-PCR assays confer sensitivity required to detect sdRNAs

Traditional RT-PCR amplification methods can lack specificity for sdRNAs that are processed from snoRNA and carry exactly the same sequence as their precursor snoRNAs. As revealed in our northern blot hybridization assays, total RNA pool contains high amounts of full-length snoRNAs but none of tested sdRNAs were detected with this technique (Fig. 1). To investigate the ability of stem-loop RT-PCR assays to detect only short sdRNAs but not the full-length snoRNAs, the reactions were performed with LMW RNA (LMW, 10-60 nt) purified from total RNA. Based on polyacrylamide gel electrophoresis results (Fig. 1, left panel), we decided to use Trizol-isolated RNAs for size separation since this RNA pool was visibly enriched in small RNA fraction.

To establish the sensitivity of stem-loop RT-PCR, a step-wise dilution of LMW RNA obtained from *S. cerevisiae* were prepared. This amplification was performed in a semi-quantitative manner, using 25-31 cycles (Fig. 3). After gel separation of the reaction products, we were able to observe clear detection signals for *S. cerevisiae* 5′-sdRNA 128 from as little as 50 ng RNA after 25 cycles of PCR, 3′-sdRNA 128 from 100 ng of RNA after 25 PCR cycles, and 5′-sdRNA 67 from 100 ng of RNA after 29 PCR cycles. At this number of cycles, no amplification was obtained in RT nor PCR water controls (-RT and -PCR lanes in Fig. 3, respectively). However, 31 or more cycles of PCR gave rise to some non-specific amplification in control reactions in case of 5′-sdRNA 128 primers. These results suggest that the stem-loop RT-PCR assay provides sensitivity sufficient for detection of sdRNAs in small RNA fractions.

Discussion

Since the discoveries of the functional potential of small RNAs, there are growing number of efforts for the development of techniques characterized by improved sensitivity and specificity of small RNA detection. Current methods for the detection and quantification of small RNAs are largely based on cloning, northern blotting, primer extension, or microarrays. All of these techniques are widely used and tested, especially for microRNA profiling. For the quantification of small RNA, low sensitivity becomes a problem because it is difficult to amplify short RNA molecules. Furthermore, low specificity may lead to false positive signals from closely related RNAs, precursors, or genomic sequences. Concerning northern blot hybridization assays, which is a gold standard for RNA detection and quantification, many efforts were focused on probing techniques and RNA/membrane attachment. It has been shown that using LNAs instead of standard antisense DNA probes increases sensitivity in detecting short RNA at least 10-fold, with the same specificity to the target molecule (Válóczi et al. 2004). LNA probes allowed, for instance, to detect small amounts (~27 000 molecules/cell) of 18-mer RNA, a regulatory ribosome-associated molecule needed for stress-adaptation in *S. cerevisiae* (Pircher et al. 2014). Alternatively, instead of conventional UV-cross-linking of total RNAs on nylon detection membranes, the use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-mediated chemical cross-linking enhanced detection of small RNAs by up to 50-fold (Pal and Hamilton 2008). However, when it comes to less-abundant snoRNA-derived small RNAs, none of techniques mentioned above possess sensitivity sufficient enough for their reliable detection in *S. cerevisiae* (Fig. 1 and Fig. 2).

We have therefore decided to use a RT-PCR-based method. Because of the small size of sdRNAs, the reverse transcription reaction is initiated with a stem-loop primer that provides higher specificity and sensitivity than linear ones (Chen et al. 2005). Detection sensitivity was further increased by a pulsed RT reaction (Tang et al. 2006). Varkonyi-Gasic et al. (2007) have demonstrated a successful detection of several microRNAs from 20 pg of plant tissue total RNA. We have also successfully implemented this method and were able to detect three sdRNAs of different origin with as little as 50 ng of LMW RNAs (10-60 nt) from *S. cerevisiae*.

It has been shown that small RNA expression levels vary significantly among different species and tissues. Therefore, the reliable and sensitive quantification of small RNA expression levels in specific cellular compartments is of special importance. A number of specific quantitative RT-PCR (qRT-PCR) techniques were developed and optimized for miRNA detection, including real-time methods based upon reverse transcription (RT) reaction with a stem-loop primer followed by a TaqMan PCR ana-

lysis (Chen et al. 2005). Quantification of sdRNAs in *S. cerevisiae* with the means of qRT-PCR cannot be done because of two major reasons: 1) the processing events take place on stable, functional, and essential RNA and 2) in real-time PCR assays, there is a need for a reporter gene expression-level estimation. Concerning the processing events of the precursor snoRNA, the full-length snoRNA and its derivative sdRNA are of the same sequence. Therefore, stem-loop primer for RT reaction cannot distinguish between sdRNA and its precursor molecule. This was clearly visible in our experimental data, when we observed amplification products in both LMW and high-molecular-weight (HMW) RNA fractions (data not showed). The amplification product was derived from small sdRNA (within LMW RNA) and full-length precursor snoRNA (within HMW RNA). Similar situation takes place with the second consideration – the need for estimation of sdRNA expression levels in relation to the reporter RNA expression levels. Choosing a proper RNA with constitutive expression pattern is not a problem within the total RNA pool. However, within the total RNA, it would not be possible to distinguish between the sdRNA and their precursors. This problem could be overcome by using LMW RNA fraction instead of total RNA. Unfortunately, in *S. cerevisiae,* there is no known small RNA that could be considered as a reporter RNA for the estimation of relative gene expression levels. Considering all of the above, a stem-loop pulsed reverse transcription followed by PCR used in this study is a suitable method for sensitive detection and semi-quantification of low-abundant sdRNA expression in *S. cerevisiae*.

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