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A rapid and sensitive nonradioactive method applicable for genome-wide analysis of *Saccharomyces cerevisiae* genes involved in small RNA biology

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Abstract

The conventional small RNA isolation and detection methods for yeast cells have been designed for a small number of samples. In order to conduct a genome-wide assessment of how each gene product impacts upon small non-coding RNAs, we developed a rapid method for analyzing small RNAs from *Saccharomyces cerevisiae* wild-type and mutants cells in the deletion and temperature-sensitive (ts) collections. Our method implements three optimized techniques: a procedure for growing small yeast cultures in 96-deepwell plates, a fast procedure for small RNA isolation from the plates, and a sensitive nonradioactive Northern method for RNA detection. The RNA isolation procedure is highly reproducible and requires only 4 hours for processing 96 samples, and yields RNA of good quality and quantity. The nonradioactive Northern method employs digoxigenin (DIG)-labeled DNA probes and chemiluminescence. It detects femtomole-level small RNAs within 1-minute exposure time. We minimized the processing time for large-scale analysis and optimized the stripping and re-probing procedures for analysis of multiple RNAs from a single membrane. The method described is rapid, sensitive, safe, and cost-effective for genome-wide screens of novel genes involved in the biogenesis, subcellular trafficking, and stability of small RNAs. Moreover, it will be useful to educational laboratory class venues and to research institutions with limited access to radioisotopes or robots.

Keywords

Saccharomyces cerevisiae; genome-wide analysis; small noncoding RNAs; RNA isolation; nonradioactive Northern analysis

INTRODUCTION

Small non-coding RNAs play crucial roles in many important processes, including mRNA and rRNA processing, translation, and gene regulation (Finnegan *et al.* 2003). In addition to the major types of small RNAs that include 5S and 5.8S rRNAs, tRNAs, snRNAs, snoRNAs, and microRNAs, recent applications of deep sequencing methodologies have led to discovery of novel small RNAs (Pais *et al.* 2011; Wang *et al.* 2009). Although small RNAs have been studied for decades, there are numerous unresolved issues regarding the cell biology of both well-known and newly-identified small RNAs. For example, several

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major factors involved in the metabolism and subcellular trafficking of one of the most characterized small RNAs, tRNAs, have not been elucidated. There is an unknown nuclear export pathway(s) for intron-containing tRNAs in yeasts (Hopper *et al.* 2008; Murthi *et al.* 2010). Moreover, the molecular mechanisms for regulating tRNA processing, subcellular trafficking, and stability remain unclear (Phizicky and Hopper, 2010). Therefore, efficient methods for identifying missing players are critical for a complete understanding of small RNA metabolism and subcellular movement.

Genome-wide screens have been widely used for identifying genes of interest in the yeast, *Saccharomyces cerevisiae*. The *S. cerevisiae* deletion and temperature-sensitive collections, which together include mutations for virtually all annotated genes of the yeast proteome (Ben-Aroya *et al.* 2008; Li *et al.* 2011; Winzeler *et al.* 1999), have provided powerful tools for genome-wide screens. However, a genome-wide screen to discover novel players in the biology of a small RNA has been challenging, due to the requirement of RNA isolation and analysis of ~6200 different yeast mutants.

Isolation of small RNAs from yeast has been mostly performed by phenol extraction of unbroken cells (Hopper *et al.* 1980; Ribaudou *et al.* 2001; Rubin 1975). The methods yield RNAs of low molecular weight, such as 5S and 5.8S rRNAs, tRNAs, snRNAs, and snoRNA, excluding large RNAs such as mRNAs and 18S and 28S rRNAs. However, the methods are time-consuming and allow RNA isolation from only a small number of samples at a time. Therefore, an efficient RNA isolation protocol is needed for genome-wide analyses.

In addition to a rapid RNA isolation method, a fast and sensitive RNA detection method is indispensable for genome-wide RNA studies. RT-PCR is a frequently used method to quantify RNA abundance. However, it fails to quantify many small RNAs that are highly modified (Czerwonec *et al.* 2009; Dunin-Horkawicz *et al.* 2006), since numerous modified residues block reverse transcription (Motorin *et al.* 2007). Deep sequencing has also been utilized for measuring the expression levels of small RNAs in eukaryotic cells (Pais *et al.* 2011; Wang *et al.* 2009). However, due to the cost of the method, it is not currently feasible to perform genome-wide deep sequencing reactions for a large number of strains in the mutant collections. Hybridization-based RNA detection and quantification techniques, e.g. microarray, that employ DNA or RNA probes have been utilized in numerous studies for detection and quantification of specific RNAs. For example, an oligonucleotide tiling microarray analysis was used to assess the roles of 468 yeast genes implicated in noncoding RNA processing (Peng *et al.* 2003). However, an unbiased functional analysis of every gene in the genome genes will be of importance for a comprehensive view of the relationship between one small RNA and the whole proteome.

In addition to microarray analysis, a less technical but very powerful hybridization technique, Northern blotting, has also been a standard technique for RNA analysis. It usually involves radioactive (^{32}P) labeling of the nucleic acid probes, hybridization of the labeled probe, and detection of the radioactive signals. However, Northern blotting is labor-intensive and time-consuming, and often involves long exposure times, especially for low abundant RNAs. Moreover, the radioisotopic probes are associated with problems of safety, stability, and disposal, and therefore at many institutions the use of radioisotope is strictly regulated, which limits the radioactive Northern method for large-scale RNA analyses.

Biotin, fluorescein, or digoxigenin (Leary *et al.* 1983; Matthews *et al.* 1988) have been introduced as alternatives to radioisotopes for Northern blotting. Digoxigenin (DIG)-labeled probes coupled with a secondary enzyme-conjugated antibody and a chemiluminescent substrate have emerged as a replacement for radioisotopes for RNA detection (Dooley *et al.*

1988; Engler-Blum *et al.* 1993; Holtke *et al.* 1990; Kim *et al.* 2010; Lanzillo 1991; Ramkissoon *et al.* 2006), because the DIG labeled probes are highly stable and have no safety or disposal problems of radioisotopes.

In order to conduct a genome-wide assessment of the impact of every yeast gene upon one or multiple small RNA(s), we developed an optimized method for analyzing each strain in the yeast deletion and *ts* collections. This method consists of a procedure for growing small yeast cultures in 96-deepwell plates, followed by a fast procedure small RNA isolation from the plates and a sensitive nonradioactive Northern protocol. The detection protocol utilizes digoxigenin (DIG)-labeled DNA probes, an anti-DIG antibody and an ultrasensitive chemiluminescent substrate that detects femtomole-levels of small RNAs within as little as 1-minute exposure. Our method combining culturing cells in deep-96 well plates and optimized RNA isolation and detection procedures enables rapid genome-wide studies of yeast genes and will therefore facilitate the discovery of novel yeast gene products that function in many facets of small RNA biology, including production, function, subcellular trafficking and stability. Furthermore, this method will be also useful for genome-wide small RNA studies in undergraduate classes or research institutions where robots and/or radioisotopes are not available.

MATERIALS AND METHODS

Yeast strains and media

The yeast deletion collection was purchased from Open Biosystems, USA; a *ts* collection was a gift from Dr. Charlie Boone (Li *et al.* 2011). 1 μ L of yeast strains was transferred from the collections into polypropylene 96-deepwell plates (Eppendorf, Cat. No. 0030 502.302) containing 0.5 mL of rich media (YEPD) per well using a multichannel pipette (ePET, Cat. No. YQ20369). Plates were incubated at 23°C for 2 days on a platform shaker incubator (New Brunswick Scientific, Excella E24) set at 220 rpm. Then, 1.5 μ L of the two-day culture was used to inoculate 1.5 mL of fresh YEPD media for overnight cultures that were used for RNA extraction. The *ts* mutants were shifted to 37°C for 2 hours before RNA extraction. Throughout the incubation, the plates were placed at a 60 degree angle to ensure efficient aeration of cultures in the deep wells, and sterile breathable seal films (Excel Scientific, Cat. No. BS25) were used to cover the plates to facilitate air circulation.

96-deepwell plate yeast small RNA extraction

Small RNAs (tRNAs, 5S rRNA, 5.8S rRNA, and other small RNAs) were extracted from the cells by an optimized phenol extraction method. 1.5 mL of yeast in the 96-deepwell plates were cultured overnight to early log phase ($OD_{600} \sim 0.3$). The cells were then collected at 4°C by centrifugation (Jouan centrifuge CR412) using microtiter plate adaptors (Labrepco, Cat. No. 51232072). The supernatants were evacuated and the cell pellets were resuspended in 0.32 mL of cold TSE (0.01 M Tris, 0.01 M EDTA, 0.1 M NaCl, pH 7.5) and 0.4 mL of TSE-buffered phenol using a multichannel pipette. The deep-well plates were then sealed with a plastic seal film (Excel Scientific, Cat. No. STRSEALPLT) and vortexed by use of a heavy duty vortex mixer (Fisher Scientific, S02216109) with a microplate holder (Fisher Scientific, 02216117) for 30 sec every 3 min for a total of 20 min. The plates were incubated in 55°C water bath between vortexing. Plates were then incubated on ice for 5 min. [Note: the steps to this point can be modified for use with robotic systems.] Since we are unaware of commercially available deepwell plates able to withstand the speeds ($\sim 10,000 \times g$) required for small RNA precipitation or a centrifuge rotor that can accommodate deepwell plates for the high-speed collection of RNA precipitates, we transferred the samples from the deep-well plates to RNase-free microcentrifuge tubes. The microcentrifuge tubes were then centrifuged at $21130 \times g$ for 10 min at 4 °C. After

centrifugation, the aqueous phases were transferred individually to new RNase-free microcentrifuge tubes containing 600 μL of cold 100% EtOH and 20 μL of 3M NaOAc. Samples were transferred to -80°C for at least one half hour. At this step, the sample can be store at -80°C for months. Then small RNAs were collected from the sample by centrifugation at $21130 \times g$ for 20 min at 4°C . The supernatant was removed and the RNA pellets were washed with 70% ethanol. Then RNA pellets were air-dried and dissolved in 11 μL of RNase-free water. RNA concentration was measured by spectrophotometry at 260 nm (A260) and RNA quality was determined by assessing the A260/280 ratios. Each 1.5 mL of yeast cultures in deepwell plates usually yields 3.0–5.0 μg of small RNAs with A260/280 of 1.9–2.2.

Preparation of Northern blots: Gel Electrophoresis, Transfer and UV Cross-linking

Usually 28 samples were loaded on a 10% polyacrylamide, neutral, 8 M urea gel (16 cm $\times$ 18 cm) and small RNAs were electrophoretically separated for 20 hours at 110 V at 4°C . RNAs in the gel were transferred onto a positively charged nylon membrane (GE Healthcare, Cat. No. RPN303B) for 2 hours at 0.6 amp. RNAs were fixed to the membrane with a Spectrolinker XL-100 UV crosslinker by an energy dosage of 120 millijoules/cm².

Preparation of DIG-labeled probes

One to four DIG-11-UTP molecules were added to probes 01 and 03 by DIG-3' end tailing reaction according to the published procedures (Amberg *et al.* 1992; Sarkar *et al.* 1998). Probe 02, labeled with a single DIG-11-UTP, was synthesized by Sigma Aldrich.

Probe sequences are as listed below.

Probe 01: 5'-

GGCACAGAACTTCGGAACCGAATGTTGCTATAAGCACGAAGCTCTAACC
ACTGAGCTACA-3'

Probe 02: 5'-

GTGGGGATTGAACCCACGACGGTCGCGTTATAAGCACGAAGCTCTAACCAC
TGAGCTACA-3'

Probe 03: 5'-

GATAATTGGTATGTCTCATTTCGGAACCTCAAAGTTCCATCTGAAGTAGCAAAT
ATGTTATTAC-3'

Hybridization and detection of DIG-labeled DNA probes

After cross-linking, the membrane were placed in hybridization tubes (Wheaton, Cat. No. 1324597j). Prehybridization buffer [5 $\times$ saline-sodium citrate (SSC), 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) Roche Blocking Reagent (Cat. No. 11096176001)] was added and the membrane was incubated at 37°C for 30 min with rotation. Prehybridization buffer was replaced by hybridization buffer (prehybridization buffer containing 1 nM DIG-labeled probe) and the membrane was hybridized at 37°C overnight with rotation. After hybridization, probes in hybridization buffer can be retrieved for subsequent use; DIG-labeled probes are stable for months in -20°C and can be reused at least 10 times.

After hybridization the membrane was washed with 15 mL of 2 $\times$ SSC containing 0.1% SDS for four times (10 min $\times$ 4) at 37°C and then equilibrated in washing buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5, 0.3% Tween 20) for 2 min at room temperature (RT). Next, the membrane were incubated in blocking buffer [1% (w/v) Roche Blocking Reagent, 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5] for 30 min at RT. Upon removal of blocking buffer, the

membrane was incubated in antibody solution [1: 20,000 dilution of Anti-DIG antibody conjugated with alkaline phosphatase (Roche, Cat. No. 11093274910) in blocking buffer] for 30 min at RT. The membrane was washed twice (15 min $\times$ 2) with washing buffer.

To detect the RNAs, the membrane was equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 3 min. After removal of the detection buffer, the membrane was placed inside of a hybridization bag. The substrate solution for alkaline phosphatase [0.125mM CDP-Star, Disodium 4-chloro-3-(methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1.3, decan]-1-4-yl)phenyl phosphate, (Roche, Cat. No. 11685627001) in detection buffer] was immediately applied over the surface of the membrane which was quickly covered by the other side of the hybridization bag. The sides of the hybridization bag were then heat-sealed. Throughout the prehybridization, hybridization and detection process, the membrane was kept wet to avoid non-specific high background signals.

To detect the RNA signals, the membrane was expose to a Lumi-Imager (Boehringer Mannheim, Mannheim, Germany) and the signal intensity of each band was quantified as absolute integrated Boehringer Light Units (BLU) using a LumiAnalyst Version 3.0 software. One minute of exposure is usually sufficient to generate a strong signal. The membrane was then stored at 4 °C for the subsequent stripping and re-probing steps if needed.

Stripping and re-probing

The membrane was kept wet in hybridization bag with detection buffer. To strip the signal, the membrane was placed in a hybridization tube with 0.1% SDS at 75°C 6 times for 10 min. After stripping, the membrane was rinsed with 5x SSC at 37°C for 5 min. Prehybridization and hybridization were then carried out for re-probing with a different DIG-labeled probe in the same manner as described above.

Preparation and detection of ^{32}P -labeled probes

γ - ^{32}P -labeled probes were prepared and hybridized with RNAs as described previously (Whitney *et al.* 2007). The specific activity of the γ - ^{32}P labeled probes was 4.5 $\mu\text{Ci}/\text{pmol}$ and the radioactive signals were detected by a Typhoon Trio imager.

RESULTS AND DISCUSSION

A procedure for large-scale yeast small RNA isolation from 96-deepwell plates

We first optimized conditions for yeast cell cultures in 96-deepwell plates. Because the deletion and *ts* collections are available in the 96-well microtiter plates, we could propagate cells for RNA analyses by transferring the strains in those collections to 96-deepwell plates. Due to the high height/width ratio (44.1 mm/7.5 mm) of the deep wells, shaking of the deepwell plate horizontally failed to circulate and aerate cells. We overcame this obstacle by placing the plates at a 60-degree angle on a platform shaker incubator at 220 rpm. Moreover, in order to facilitate the air circulation in the cell culture as well as to avoid any contamination, sterile breathable seal films instead of plastic seals were used to cover the plates.

The maximum volume of each well in the 96-deepwell plates is 2 mL. Therefore, it is necessary to develop a procedure that not only allows fast simultaneous processing of 96 samples, but also yields RNAs of high quality and quantity from small cell cultures. We optimized the procedure by modifying each step of a conventional phenol extraction method that was developed for large cultures (Hopper *et al.* 1980). First, we tested whether the addition of chloroform into phenol would improve the efficiency of the RNA extraction

from small cultures. However, chloroform seemed to cause inconsistent RNA concentrations and quality (A260/A280 ratios) from identical cultures (data not shown). We next shortened the incubation time in phenol by increasing the incubation temperature. Incubating at 55 °C for 20 min with vortex between incubations produced equivalent quantities and quality of RNA as the conventional 1-hour incubation at 37 °C (data not shown). Moreover, the time for incubating samples on ice was reduced to 5 min without affecting the isolation efficiency (data not shown). Furthermore, although RNA isolation procedures from larger cultures (e.g. 15 mL) generally employ two phenol extraction steps to remove protein contaminants, we found that a single phenol extraction is sufficient to yield RNA of good quality from the small cultures. By modifying every step as described, the extraction of small RNAs from 96 strains was shortened to 4 hours.

We examined the quality of the RNA extracted with the optimized procedure from the deepwell cultures of 30 different strains that were randomly chosen from the yeast deletion and *ts* collections. The ratios of A260/280 ranged from 1.90 to 2.20 with an average of 1.94. The reproducibility of the optimized procedure was also tested using triplicate extractions from the same strains. The average standard deviation of the A260/280 generated from the triplicates of 3 different strains is 0.0088, showing high reproducibility. Therefore, a fast and highly reproducible procedure for large-scale isolation of yeast small RNAs from 96-deepwell plates has been established (Figure 1A).

We compared the RNAs isolated by our optimized method with RNAs isolated by the conventional method utilizing EtBr staining and Northern analysis using radioactive probes specific for tRNA^{Ile}_{UAU} (Figure 1B). The electrophoresis of RNAs isolated by the optimized method and the conventional method show the same RNA profiles, including the prominent 5.8S and 5S ribosomal RNAs, tRNAs and other small RNAs (Figure 1C, left). tRNA^{Ile}_{UAU} is encoded by two identical tRNA genes that contain the longest yeast tRNA intron (60 nt). To detect tRNA^{Ile}_{UAU}, a 62 nt oligonucleotide probe (probe 01) complementary to the majority of the 5' exon and half of the intron (Figure 1B) was designed. This oligonucleotide detects primary tRNA transcripts (P), end-matured intron-containing tRNAs (I), and mature modified tRNAs (M) as well as other intermediates described below. Northern analysis of tRNA^{Ile}_{UAU} isolated from 15 mL cultures of wild-type (wt) cells by conventional phenol extraction procedures detected the P, I, and M tRNAs (Figure 1C, right). Since only half of the probe is complementary to mature tRNA^{Ile} low levels of the M species are detected. As expected (Hopper *et al.* 1980; Murthi *et al.* 2010), RNAs isolated from *los1Δ* cells, in which the tRNA exportin Los1 is deleted, show accumulation of the end-matured intron-containing tRNAs (Figure 1C, right). Remarkably, the Northern profiles of RNAs extracted from wt and *los1Δ* cells cultured in deepwell plates utilizing the rapid optimized procedure are identical to the RNA profiles from the conventional procedures (Figure 1C). We conclude that the optimized RNA isolation procedure is suitable for accurate genome-wide analyses of RNAs isolated from small cultures.

Assessment of the sensitivity of DIG-labeled DNA probes and optimization of DIG Northern protocol for genome-wide analyses

To enhance detection of small RNAs in ~6200 yeast mutants, we sought to develop a simple, fast, cost-effective, and nonradioactive protocol for Northern analysis. To this end, we employed DIG-labeled probes. First, we determined the sensitivity of DIG-labeled probes by immune-dot blot assays. Probe 01 was labeled with DIG-11-dUTP at its 3' end by utilizing terminal deoxynucleotidyl transferase. This labeling reaction results in addition of one to four digoxigenin-11-UTP molecules to each oligonucleotide (Amberg *et al.* 1992; Sarkar *et al.* 1998). We applied serial dilutions of this probe onto a positively charged Nylon membrane and fixed the probe on the membrane by UV crosslinking. The probe was then

detected by use of an anti-DIG antibody and a chemiluminescent substrate. Chemiluminescence is highly sensitive as one femtomole of probe could be detected after one-min exposure (Figure 2). We also examined a commercially purchased tRNA^{Ile} probe (probe 02) labeled with a single DIG-11-UTP molecule (Sigma Aldrich). Three femtomoles of probe 02 generated similar intensity to one femtomole of probe 01, consistent with the number of DIG-11-UTP introduced to the probes (Figure 2). Thus, DIG-labeled oligonucleotide probes detect femtomole-level RNAs within 1 min.

We next sought to develop an optimized Northern hybridization and detection protocol utilizing the DIG-labeled probes. Since the Roche hybridization buffer was reported to result in lower nonspecific background signals than other commercially available buffers (Kim *et al.* 2010), we compared it with the Denhardt's hybridization buffer that is widely used for ³²P Northern analyses. Denhardt's buffer resulted in strong nonspecific background signals while the Roche hybridization buffer yielded almost no background signal (data not shown). Therefore, the Roche hybridization buffer was chosen for the hybridization of DIG-labeled probes. We also optimized the steps for blocking the membrane, binding of the antibody, washing the membrane, and detection of chemiluminescent substrate in order to shorten the time for analysis and to reduce reagent costs (see Materials and Methods).

We examined the performance of DIG-labeled probes and chemiluminescence in the linear detection of tRNA^{Ile} metabolic intermediates for RNAs extracted from wt cells propagated in the 96-deepwell plates and isolated by our optimized procedures. The DIG method generated detectable signals for both primary tRNA^{Ile} transcript and end-processed intron-containing tRNA^{Ile} from as little as 1.0 µg of total small RNA after exposure for 30 sec (Figure 3A). The signal intensity of primary tRNA^{Ile} transcript at 30 sec is rather linear as the amount of RNA load increases from 1.0 to 7.0 µg (Line 1 in Figure 3B). Longer exposure of 1 or 3 min generated much stronger signals but still showed the same quantification results (I/P ratio in Figure 3A). Moreover, longer exposure still yields a linear increase in the signals as the amount of RNA increased (Figure 3B, lines 2 and 3).

We also examined the performance of DIG chemiluminescent Northern procedure in detecting another low abundant small RNA in yeast, snR24. snR24 is a C/D box small nucleolar RNA; it was estimated that its cellular content in *S. cerevisiae* is 70–160 molecules per cell (Qu *et al.* 1995). A 62 nt DIG-labeled probe (probe 03) was used to detect snR24 (Figure 3C) from increasing amount of total small RNAs, 1.0, 3.0, 5.0, or 7.0 µg. The DIG chemiluminescent Northern yielded detectable signals of snR24 after exposure of 1.5 min. Stronger signals were generated after 3 min and 4.5 min exposure and the quantification of signal intensity shows linear detection of snR24 (Figure 3D). We calculate that this method can easily detect less than 6.6×10^8 snR24 molecules (1 femtomole of this RNA species). Since 3.0–5.0 µg of small RNAs were usually yielded from each 1.5 mL of yeast cultures, the data show that the DIG chemiluminescence method is extremely fast and sensitive for the detection of small RNAs isolated from the 96-deepwell plates and is therefore ideal for genome-wide RNA analyses.

Comparison of DIG chemiluminescent with ³²P Northern method

We compared the DIG chemiluminescent Northern method with the conventional isotopic Northern method for detection of tRNA processing intermediates. We chose to study tRNA^{Ile} in wt and three mutant strains defective in tRNA trafficking or processing—*los1Δ* which accumulates end-matured intron containing tRNAs (I) (Hopper *et al.* 1980; Murthi *et al.* 2010), *sen2-42* which accumulates I and 5' 2/3 tRNA (Yoshihisa *et al.* 2003; Trotta *et al.* 1997), and *rlg1-4* cells which accumulates tRNA halves (Phizicky *et al.* 1992). 3.0 µg of small RNAs were loaded into each lane and the DIG-labeled probe 01 or ³²P-labeled probe were employed for hybridization. As shown in Figure 4, 30 sec exposure of a

chemiluminescent Northern blot yields comparable sensitivity to that of 16 hr exposure of the isotopic Northern blot for all tRNA intermediates in each strain tested. 3 min exposure of DIG Northern blot yielded much stronger signal than radioactive method and resulted in better detection of the 5' 2/3 tRNA^{Leu} accumulation in *sen2-42* and the 5' half accumulation in *rlg1-4* at the nonpermissive temperature. The quantification data (I/P ratios) of the DIG chemiluminescent Northern blot is nearly identical to that of the ³²P Northern blot. The advantage of DIG chemiluminescent method in tRNA detection is significant considering that the DIG-probe concentration is 1/10 of the radioactive probe and the exposure time is much shorter. We anticipate that the method will facilitate large-scale screens aiming to uncover yeast mutants in the deletion and ts collections that affect the processing, subcellular movement, or stability of tRNA or other small RNAs.

For several reasons we believe the described method will have advantages for other types of analyses. First, the cell culture and RNA isolation procedure may be applied to other model organisms like *Schizosaccharomyces pombe*. Second, we found that the chemiluminescent Northern method, most importantly the design of DIG-labeled probes, is adaptable to the detection of any other small non-coding RNAs as well as mRNAs. Third, the RNA isolation procedure can be easily modified to perform large-scale mRNA isolation by increasing the temperature and the length of time for incubation of cells in hot phenol. Fourth, the method can be adapted to use of robots for high-throughput performance. Finally, the method will enable educational programs or research institutions that have limited access to robots or radioisotope to conduct RNA analysis. In conclusion, this method provides a fast and sensitive approach for genome-wide analysis of all yeast genes in small RNA metabolism and will promote the discovery of novel yeast gene products that function in the biogenesis and cell biology of small RNAs.

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Figure 1.

96-deepwell plate yeast cell culture and small RNA isolation procedure.

(A) Comparison of the conventional small RNA isolation procedure and the 96-deepwell plate small RNA isolation procedure. The minimum time for processing 96 samples using the optimized method is significantly less than using conventional procedures. (B) Probe 01 which is complementary to 5' exon and intron junction was designed to detect tRNA^{Ile} processing intermediates by Northern analysis. (C) EtBr staining (left) and Northern analysis (right) of RNAs isolated by the conventional procedure and the optimized 96-deepwell plate procedure shows the same patterns of RNAs. 4.0 μ g of total small RNAs isolated from wt and *los1Δ* cells were loaded on to each lane and resolved through a 10% polyacrylamide gel. Staining of the whole gel (left) shows the prominent 5.8S and 5S ribosomal RNAs and tRNAs. RNAs in this gel were transferred to a membrane and tRNA^{Ile} processing intermediates were detected by Northern analysis (right) using γ -³²P-labeled Probe 01. (P), primary tRNA transcript with 5' and 3' ends and intron; (I), end-matured intron-containing tRNA; (M), intronless mature tRNA. The blot was exposed for 16 hours for ³²P detection.

Figure 2. Detection of the DIG-labeled DNA probes by dot blot shows femtomole-level sensitivity. Probe 01 was labeled with one to four DIG-11-UTP molecules by 3' end labeling reaction; probe 02 with only one DIG-11-UTP molecule was synthesized by Sigma Aldrich. 1 μ l of a serial dilutions (1–1000 fmol/ μ l) of each probe was blotted and blots were exposed for 1 and 2 min using a Roche Lumi Imager.

Figure 3.

Linear and sensitive detection of the DIG chemiluminescent Northern blot. (A) Detection of tRNA^{Ile} from increasing amounts of small RNAs (1.0, 3.0, 5.0, and 7.0 µg). Hybridization was carried out with DIG-labeled probe 01 and the blot was exposed for various amount of time (30 sec, 1 min, and 3 min). Ratios of the signal intensities of the primary tRNA transcript (P) and end-matured intron-containing tRNA (I) are shown. (B) The signal intensity (demonstrated as Boehringer light unit, BLU) of primary tRNA transcript (P) is proportional to the amount of RNAs loaded after different exposure time (30 sec, 1 min or 3 min). The coefficient of determination R^2 value for Line 1, Line 2, and Line 3 are 0.98, 0.99, and 1.0 respectively. (C) Detection of snR24 from increasing amounts of small RNAs (1.0, 3.0, 5.0, and 7.0 µg). Hybridization was carried out with DIG-labeled probe 03 and the blot was exposed for various amount of time (1.5 min, 3 min, and 4.5 min). (D) The signal intensity of snR24 is proportional to the amount of RNAs loaded after different exposure time (1.5 min, 3 min or 4.5 min). The coefficient of determination, R^2 value, for Line 1, Line 2, and Line 3 are 1.0, 1.0, and 1.0 respectively.

Figure 4.

Comparison of the performances of DIG-based and ³²P-based Northern methods for detection of tRNA intermediates. Wt, *los1Δ*, *sen2-42*, and *rlg1-4* cells were grown in 96-deepwell plates at permissive temperature (23 °C) or after incubation for 2 hr at the non-permissive temperature (37°C). Small RNAs were isolated by our optimized 96-deepwell plate small RNA isolation procedure. 3.0 μg of RNAs were loaded to each lane. DIG-labeled or ³²P-labeled probe 01 was used to detect tRNA^{Ile} processing intermediates. The DIG Northern blot was exposed for 30 seconds (A) and 3 minutes (B), and the ³²P blot was exposed for 16 hours (C). (P) Primary tRNA transcript; (I) end-matured intron-containing tRNA; (5' 2/3) 5' 2/3 molecule consisting of 5' exon and intron; (M) intronless mature tRNA; (H) 5' tRNA half; (5S rRNA) loading control. Ratios of the signal intensities of I/P are shown.