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Identification of RNase-resistant RNAs in *Saccharomyces cerevisiae* extracts: separation from chromosomal DNA by selective precipitation

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Abstract

High quality chromosomal DNA is a requirement for many biochemical and molecular biological techniques. To isolate cellular DNA, standard protocols typically lyse cells and separate nucleic acids from other biological molecules using a combination of chemical and physical methods. After using a standard chemical-based protocol to isolate chromosomal DNA from *Saccharomyces cerevisiae* and then treatment with RNase A to degrade RNA, two RNase-resistant bands persisted when analyzed using gel electrophoresis. Interestingly, such resistant bands did not appear in preparations of *E. coli* bacterial DNA after RNase treatment. Several enzymatic, chemical and physical methods were employed in an effort to remove the resistant RNAs, including use of multiple RNases and alcohol precipitation, base hydrolysis and chromatographic methods. These experiments resulted in the development of a new method to isolate *S. cerevisiae* chromosomal DNA. This method utilizes selective precipitation of DNA in the presence of a potassium acetate/isopropanol mixture and produces high yields of chromosomal DNA without detectable contaminating RNAs.

Keywords

RNA; DNA; purification; extraction; precipitation; RNase

Introduction

Many different approaches have been developed for the extraction of nucleic acids from prokaryotic and eukaryotic cells. Such methods typically entail lysis of cells using some combination of physical and/or chemical methods, followed by separation of the nucleic acids from other biomolecules [1,2]. For example, chemical-based protocols utilize detergents and/or other chemical bond-breaking agents to lyse cells, while physical

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approaches may involve the disruption of cells using techniques such as sonication, boiling, repetitive freeze-thawing, agitation in the presence of small beads, or grinding of frozen tissues [1-4].

Protocols have also been described for the purification of specific types of nucleic acids from cells, e.g., isolation of pure DNA that does not contain RNA and vice versa [1,2,5-7]. DNA isolation schemes may be further subdivided into those methods designed primarily for purification of large chromosomal DNA fragments (e.g., to be used for subsequent polymerase chain reaction (PCR) assays or Southern blots, etc.) and those developed for purification of other molecules such as small circular plasmids, viral DNAs, or mitochondrial genomes. Development of plasmid DNA isolation methods has been of particular interest, in large part because these DNAs have become essential tools for molecular biologists, but also because of new requirements in the burgeoning field of gene therapy. Circular DNAs to be used for clinical purposes must meet a high standard of purity, meaning that robust methods for removal of contaminating RNAs, proteins and other biomolecules must be employed [8-11].

Because of heterogeneity among cells, especially differences in cell membrane and/or cell wall structures that can affect the yield and purity of extracted DNAs, many organism- and tissue-specific DNA purification procedures have been described. For example, a number of specific protocols have been described for the isolation of chromosomal, plasmid and mitochondrial DNAs from cells of the widely studied model eukaryote *Saccharomyces cerevisiae* (budding yeast) [12-23]. These methods typically involve an ordered series of steps: chemical or physical lysis of cells to release intracellular nucleic acids, sedimentation and removal of cell debris and proteins, concentration/purification of DNA molecules by precipitation with ethanol or isopropanol, and degradation of contaminating RNAs using RNase enzymes. RNases used in these procedures, as well as in many other eukaryotic and prokaryotic DNA purification methods, are single-stranded RNA (ssRNA) endonucleases such as RNase A or RNase I. Commercially available bovine RNase A preferentially breaks phosphodiester linkages at the 3′ ends of pyrimidines, while *E. coli* RNase I cleaves at the 3′ ends of all four bases [2,24,25]. An inexpensive recombinant version of the latter enzyme, called RNase I_f, has recently become available commercially and is also widely used.

As part of a series of experiments involving purification of DNA from *S. cerevisiae* cells for use in Southern blots and for studies aimed at separating chromosomal from plasmid DNA [22], we consistently observed that the yeast DNA preps contained contaminating RNAs that were resistant to RNase A digestion. These RNAs were distinctly different from those present in *E. coli* plasmid and chromosomal DNA preps, which are readily digested down to mono- and oligonucleotides by RNase A. Simple ideas such as increasing RNase enzyme concentration or extending incubation times proved ineffective. Similarly, many other straightforward approaches did not work, including use of alternative RNase enzymes and higher temperatures, base hydrolysis at high pH for selective destruction of RNA but not DNA, RNA sedimentation with lithium chloride, and the use of commercially available anion exchange and gel chromatography spin columns. Each of these procedures produced poor results, either due to inefficient removal of the contaminating RNAs or because both DNAs and RNAs were removed from the preparations. Systematic modification and testing

of an alternative idea involving sedimentation of DNA in the presence of potassium acetate and isopropanol[26] led to the development of an effective approach. Small RNAs and RNase-resistant RNA molecules in yeast chromosomal DNA preparations could be selectively removed in a single step, without concomitant loss of DNA, using the simple method described herein.

Materials and Methods

Reagents and enzymes

Tris base, ethylenediaminetetraacetic acid (EDTA), potassium acetate (KoAc), sodium acetate (NaOAc) and lysozyme were purchased from Sigma-Aldrich. TAE running buffer (50×) was purchased from Omega Bio-Tek. Sodium hydroxide (NaOH) was obtained from EM Science. Boric acid was purchased from JT Baker. Sodium dodecyl sulfate (SDS) was purchased from Mallinckrodt Baker. RNase A/T1 Cocktail Mix and RNase V1 were purchased from Invitrogen/Life Technologies. RNase I_f, 2-log DNA ladder, and dsRNA ladder were purchased from New England Biolabs. RNase A was obtained from Thermo Scientific.

Chromosomal DNA purification from yeast cells

Haploid yeast strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was used for DNA purification experiments. Chromosomal DNA was purified using the SDS lysis method as described by Lee *et al* [22]. Briefly, 4 mL of yeast cells were shaken in YPDA broth overnight at 30 °C. Three mL of cells (1.5 mL twice) were centrifuged for 10 s at 16,000 × g. The pellet was resuspended in 0.3 mL SET (6% SDS + 10 mM EDTA + 30 mM Tris, pH 8.0) solution, incubated at 65 °C for 15 min and then transferred to ice for 5 min. A total of 0.15 mL 3 M KOAc was then added to the sample. The tube was spun for 10 min at 21,000 × g, the supernatant was transferred to a new microcentrifuge tube and 0.5 mL isopropanol was mixed into the sample. The tube was then centrifuged for 1 min and the supernatant removed. The DNA pellet was washed with 0.5 mL 70% EtOH and subsequently dried for 10 min using a speedvac. The resulting pellet was resuspended in 50 μL TE. After the DNA dissolved, the solution was spun at 21,000 × g for 2 min to remove any remaining insoluble materials and the supernatant was transferred to a new tube. All electrophoresis experiments were performed using 11 cm × 14 cm Horizon gel rigs (Labrepco) and either a Thermo EC EC105 power supply or an Amersham/GE Healthcare EPS 601 power supply. A_{260}/A_{280} absorbance ratios were determined using a Nanodrop 2000 UV-Vis spectrophotometer from Thermo Scientific.

Chromosomal DNA purification from bacterial cells

DH5α *E. coli* cells were used to isolate bacterial chromosomal DNA. The cells were shaken in 3 mL TB broth at 37 °C overnight. A total of 1.5 mL cells were centrifuged and resuspended in 150 μL lysis solution (6% SDS, 10 mM EDTA, 30 mM Tris, pH 8.0). Subsequently, 25 μL lysozyme solution (10 mg/mL dissolved in 25 mM Tris, pH 8.0) was added and the sample was incubated at room temperature for 3 min, followed by a 5 min incubation at 65 °C. After the incubation period, 150 μL of 3 M KOAc was added. The solution was then spun for 10 min at 21,000 × g and the supernatant was transferred to a new

microcentrifuge tube. The DNA was precipitated by adding 500 μL isopropanol followed by a 1 min centrifugation. The pellet was washed with 500 μL cold 70% EtOH and dried for 10 min using a speedvac. The pellet was resuspended in 50 μL TE. RNA remaining in the preparation was enzymatically degraded with addition of 2 μL RNase A (1 mg/mL) and incubation at 37 °C for 15 min.

RNase digestion and Ethanol Precipitation

Purified yeast chromosomal DNA was subjected to RNA digestion with the addition of RNase A to a final concentration of 0.04 μg/μL. Typically, this involved addition of 2 μL of 1 mg/mL RNase (in 50% glycerol) to 50 μl of DNA. The yeast DNA/RNA solutions were then incubated at 37 °C for 15 min. Samples were visualized for purity via gel electrophoresis on a 1.2% agarose gel run in 0.5× TB (45 mM Tris, 45 mM borate) at 300 V for 15 minutes as described [27]. High voltages and short run times were used to minimize diffusion of small RNAs.

The typical size of contaminating RNA oligonucleotides retained after RNase A or I_f treatment of chromosomal DNA preps was determined by calculating the migration distances of the RNA bands relative to a dsRNA ladder on a 3.5% agarose gel run at 300 V for 15 min in 0.5× TB buffer. A bestfit trendline and equation derived from a plot of log DNA size versus distance travelled were calculated using Microsoft Excel.

A series of reactions were performed on BY4742 chromosomal DNA preps with contaminating RNA including: the addition of RNase Cocktail (24 U/ml RNase A and 1.0 U/μl RNase T1 final concentrations), RNase V1 (5 U/ml) without buffer, RNase V1 (5 U/ml) in 1× RNA Structure buffer, RNase Cocktail (24 U/ml RNase A and 1.0 U/μl RNase T1) plus RNase V1 (5 U/ml) together without buffer, and RNase V1 (5 U/ml) plus RNase Cocktail in 1× RNA Structure buffer. Resulting reaction mixtures were incubated at 37 °C for 30 min.

Ethanol precipitation was performed by adding 0.1 volume of 3 M NaOAc and 2.5 volumes of 100% EtOH to 50 μL of the RNased treated sample. The sample was mixed and spun for 5 min at 21,000 × g. The supernatant was discarded and 500 μL of 70% EtOH was used to wash the DNA pellet. The sample was re-spun for 3 min and dried for 10 min using a speedvac. The DNA pellet was resuspended in the original volume of TE.

Base hydrolysis of contaminating RNAs

Base hydrolysis using either sodium hydroxide or potassium hydroxide at 0.2 M and 0.3 M final concentrations was tested to remove contaminating RNAs. The effect of EDTA on the hydrolysis reaction was explored by varying its concentration from 0 - 0.25 M. Once the pH was raised with NaOH or KOH, the samples were allowed to react for 15 min at either room temperature or 65 °C. All reaction conditions tested resulted in elimination of the two resistant bands present after digestion with RNase A or RNase I. However, each treatment also led to poor recovery and aberrant electrophoretic migration of the resulting chromosomal DNA.

Use of columns to remove contaminating RNAs

Further attempts to remove RNA from chromosomal DNA utilized QIAprep Spin Miniprep Kit columns and the protocol provided by Qiagen, Inc. for cleaning up DNA solutions. CHROMA SPIN-1000 columns were tested using the protocol provided by Clontech Laboratories, Inc.

Optimized protocol for RNA removal from chromosomal DNA minipreps

Feliciello and Chinalli published a method for the removal of *E. coli* RNAs and proteins from plasmid DNA preparations [26]. This protocol involved the addition of 1.2 volumes of an 88% isopropanol/0.2 M KOAc mixture to a DNA solution, followed by centrifugation to selectively precipitate the DNA. Use of this method in the current study consistently resulted in only partial removal of yeast RNAs. The original method was made more stringent by testing different KOAc concentrations, different centrifugation times, and different volumes of the 88% isopropanol/KOAc mixture added to the DNA. The method could be made more efficient by reducing the concentration of KOAc and by decreasing the volume of isopropanol/KOAc mixture that was added.

The final optimized protocol for the quick removal of contaminating RNAs from yeast chromosomal DNA minipreps is as follows. Chromosomal DNA was purified from 3 mL of yeast cells grown overnight in YPDA broth and resuspended in 50 μL of TE without RNase as previously described [22]. The purified chromosomal DNA was then diluted by a factor of 4 with sterile ddH_2O, 5 μL of 1 mg/mL RNase A was added, and tubes were incubated at 37 °C for 15 min. The DNA was then selectively precipitated by adding 0.6 volumes of 88% isopropanol/0.1 M KOAc, mixing briefly, incubating at room temperature for 10 min, and spinning at 16,000 × g for 2 min. The supernatant was discarded. The DNA pellet was washed with 500 μL 70% cold EtOH and spun once again for 1 min. The supernatant was removed and the pellet was dried in a speedvac for 10 min. The chromosomal DNA pellet was then resuspended in 50 μL TE again. The method works well with modestly smaller or larger volumes and concentrations of DNA.

Results

While performing yeast chromosomal DNA isolations according to a previously described method[22], we noticed an anomaly in the chromosomal DNA preparations at the end of the procedure. This chemical-based method of DNA isolation is similar to other published protocols and depends on the lysis of cells using SDS treatment at 65°C, which is followed by protein precipitation with potassium acetate, nucleic acid precipitation using isopropanol, and resuspension of the DNA in TE [22]. Specifically, 3.0 mL of cells from YPDA broth cultures shaken overnight at 30°C were pelleted, lysed and the final purified nucleic acids were resuspended in 50 μL of TE. Running 4 μL of the 50 μL mixtures on a 1% agarose gel produced bands typical of total nucleic acid preparations obtained from prokaryotic and eukaryotic cells, i.e., a prominent band was observed above 10,000 bp in size (~ 50,000 bp), which is composed of large linear fragments generated by random shearing of chromosomal DNA during the purification procedure (Figure 1A). Below 10,000 bp, a complex mixture of

RNA bands was observed, corresponding to rRNAs, tRNAs and mRNAs, as well as many small functional RNAs.

When pure DNA is the desired nucleic acid, it is standard practice to treat preparations with an RNase enzyme, usually RNase A or RNase I (or the variant I_f), to digest the RNAs down to mononucleotides and small oligonucleotides [2]. When the RNase treated DNA is subsequently run on an agarose gel, the digested RNAs produce either no band or a small, diffuse, and weakly-stained RNA oligonucleotide band at the bottom of the gel. This is in part due to the fact that ethidium bromide stains small single-stranded nucleic acids poorly and is also a result of the rapid diffusion of the small RNAs during electrophoresis, which spreads out the fluorescent signal [27]. However, using a standard method (addition of 2 μL of 1 mg/ml RNase A to a 50 μL sample followed by incubation at 37°C for 15 minutes) on *Saccharomyces cerevisiae* DNA isolations consistently resulted in two RNase A-resistant bands in addition to the chromosomal DNA band (Figure 1B). This was unexpected and distinctly different from results seen when RNAs present in chromosomal DNA prepared from *E. coli* cells were digested with RNase A (Figure 1C). Since RNase A is a single-stranded RNA-specific enzyme, the results imply that some RNAs within yeast cells retain stable secondary or tertiary structures that cannot be cleaved by the enzyme.

To further characterize these RNase-resistant fragments, we isolated chromosomal DNA and determined the approximate sizes of the fragments using gel electrophoresis. Because of their small size, we employed a 3.5% agarose gel in which fragments ranging from approximately 5 bp to over 500 bp can be visualized using ethidium bromide [2] (Figure 2A). These gels were run at high voltage in 0.5 × TB buffer for a minimal amount of time in order to limit diffusion of small fragments as previously described [27]. Using a dsRNA ladder, we constructed a standard curve relating the sizes of the fragments to their migration distances in the gel. This plot produced a linear relationship from which we were able to determine the sizes of the RNase-resistant fragments (Figure 2B). The upper band's higher molecular weight population was more heterogeneous in size and ranged from 24-56 bp in length, while the lower band's smaller molecular weight fragments ranged from 10-16 bp in length.

The presence of these small RNase-resistant fragments may potentially affect many molecular biology experiments, e.g., the RNA fragments absorb ultraviolet light at 260 nm and can significantly hinder accurate DNA concentration determination. They may also confound downstream experiments involving enzymes that can act on both RNA and DNA. Several simple approaches were pursued in an attempt to eliminate the resistant yeast RNAs, including (a) addition of up to 10 times the normal amount of RNase A, (b) switching to RNase I_f, another commonly used single-stranded RNA nuclease that cleaves after all 4 bases, (c) increasing RNase A incubation time from 15 minutes to 2 hours, and (d) precipitation using standard ethanol or isopropanol precipitation methods after RNase treatment, which is known to sediment larger nucleic acids more efficiently than smaller molecules [2]. None of these straightforward approaches were successful (data not shown). Therefore, we sought to improve the efficiency of removal of the resistant RNAs from chromosomal DNA by testing a series of more rigorous enzymatic and physical methods.

Since folding of these fragments into double-stranded RNAs was likely to be protecting them from RNase A cleavage, the samples were heated to denature secondary structures at temperatures ranging from 50°C to 75°C for 5 minutes and then treated with RNase A according to the standard protocol. This modification resulted in small changes in the distribution of resistant fragments, primarily in the upper band, but overall did not result in removal of either band. A representative experiment involving preincubation at 65°C is shown in Figure 3A. After treatment with an 'RNase cocktail' (Invitrogen) containing RNase A and RNase T1, the large fragment appeared to be degraded while the smaller fragment remained intact (Figure 3B, lanes 2 and 3). RNase T1 also targets single-stranded RNA but differs from RNase A in that it cleaves after ribonucleotides containing G [25]. Similarly poor results were observed when RNase A and RNase I_f were employed together (data not shown).

RNase VI is an enzyme that cleaves double-stranded RNA nonspecifically [25] and is therefore potentially capable of digesting small yeast RNAs containing secondary and tertiary structures. Treatment with this enzyme did eliminate the majority of the two resistant RNA bands, but unfortunately it also consistently resulted in partial digestion of the double-stranded chromosomal DNA fragments too, indicating that it can cleave both dsRNA and dsDNA (Figure 3B, lane 4). In addition, the degradation of the DNA resulted in a significant reduction in the overall yield of the DNA thus lower both the quality and quantity of isolated DNA.

Since enzymatic methods had been unsuccessful, we tested two commercially available column-based protocols that have been optimized to isolate plasmid DNA. The first approach used Qiaprep Spin Miniprep Kit columns, which separate nucleic acids by anion-exchange. Larger nucleic acids remain bound to the column, while smaller fragments are not retained efficiently. The second approach involved CHROMA SPIN-1000 columns, which contain a proprietary gel chromatography resin that fractionates molecules based on size. For each method, two independent DNA purifications were performed (Figure 3C and 3D). In both cases, the smaller fragments were removed efficiently, but the larger fragments persisted. In the case of the Qiaprep column (Figure 3C, lanes 2 and 3), there was also a significant loss of chromosomal DNA. These results indicate that neither method is suitable for removal of the resistant RNAs from the chromosomal DNA.

Because of the susceptibility of RNA, but not DNA, to base hydrolysis [28,29], we attempted to use this chemical susceptibility to degrade the small RNA fragments. Chromosomal DNA preps were incubated with RNase A using the standard protocol and this was followed by ethanol precipitation (addition of 0.1 volume of 3 M NaOAc and 2.5 volumes of cold ethanol, followed by centrifugation and resuspension in ddH_2O; see Methods). The resulting chromosomal DNA was reacted with 0.2 M or 0.3 M sodium hydroxide based on previously described protocols[28,29]. While the small RNA fragments were removed, the overall yield of the chromosomal DNA was significantly depleted and the treated DNA migrated unusually slowly compared to the control DNA (Figure 4, lanes 3 and 4). Several trials that varied the base (sodium hydroxide or potassium hydroxide) and the reaction temperature, 25 or 65 °C, showed similar results (data not shown).

The next experiments addressed whether the RNA fragments could be separated from the chromosomal DNA using alcohol precipitation methods. Standard ethanol plus sodium acetate precipitation (Figure 4, lane 2) as well as lithium chloride precipitation (data not shown) had been unsuccessful in isolating the chromosomal DNA away from the RNA fragments. An isopropanol plus KOAc method published by Feliciello and Chinali [26] was assessed as well. This method involves the addition of 1.2 volumes of an 88% isopropanol/0.2 M KOAc mixture to a DNA solution, followed by incubation for 10 minutes at room temperature and centrifugation for 5 minutes in a microcentrifuge. Initial experiments with this method produced results that were similar to the other isolation methods that had been tested in that the two RNA bands remained. We therefore sought to improve this method. We varied the volumes of isopropanol/KOAc that were added to the samples; a representative experiment is shown in Figure 5A, which indicated that it was possible to add 0.6 volumes rather than 1.2 volumes of isopropanol/KOAc and get removal of most of the RNAs (compare lane 3 vs. lanes 1 and 2). After testing many variables, including centrifugation times, incubation times, and concentrations of isopropanol and KOAc, we developed a new improved protocol that selectively removes the RNase-resistant fragments from chromosomal DNA minipreps while not reducing the yield of chromosomal DNA.

In the final optimized method, chromosomal DNA prepared from 3 mL overnight cultures and suspended in 50 L TE (or ddH_2O) is diluted by a factor of 4 into ddH_2O and then treated with 5 μL of 1 mg/ml RNase A for 15 minutes at 37 °C. The chromosomal DNA is then selectively precipitated by adding 0.6 volumes of an 88% isopropanol/0.1 M KOAc mixture and incubating at room temperature for 10 minutes followed by centrifugation at 16,000 × g for 2 minutes. The supernatant is then discarded and the DNA pellet washed with 70% ethanol, dried in a speedvac and resuspended in the original volume of TE or ddH_2O. As shown in Figure 5B, lane 2, the RNase-resistant fragments are completely removed using this method without the quantity of the chromosomal DNA being affected. The quality of the DNA was tested by measuring the A_{260}/A_{280} ratio of chromosomal DNA extracted using the new method vs the original RNase A-treated DNA. DNAs from five separate yeast chromosomal DNA minipreps which still retained the two resistant RNA bands after RNase A treatment exhibited an A_{260}/A_{280} ratio of 2.17 +/- 0.07 (average +/- standard deviation). Five separate yeast chromosomal DNA preps isolated using the new method had an average A_{260}/A_{280} ratio of 1.87 +/- 0.18. The reduction in the A_{260}/A_{280} ratio indicates that strongly UV-absorbing RNA fragments have been removed and that the remaining DNA is highly pure with very little contaminating RNA.

Discussion

The isolation of chromosomal DNA from yeast cells has been accomplished historically using many different approaches and typically involves a final step involving degradation of contaminating RNAs using RNases. For many downstream experiments, both the quantity and quality of the isolated chromosomal DNA are critical. As part of a series of chromosomal DNA purifications from *S. cerevisiae* cells, we observed that the isolated DNA retained two small RNase-resistant bands when analyzed by gel electrophoresis. To further understand the nature of these RNase-resistant fragments, the sizes of the fragments

were determined and found to range between 10-16 bp and 24-56 bp for the lower and upper bands, respectively. The identities of the fragments are not known. Our observation that they could be eliminated by base hydrolysis in NaOH or KOH indicates that they are composed of RNAs without any apparent small DNAs. The resistant fragments could also be degraded by treatment with RNase V1, a nuclease with strong preference for double-stranded RNA that has slight cross-reactivity against double-stranded DNA (Figure 3B). These results suggest that the resistant bands are composed of unusually stable double-stranded RNA molecules. Such resistant RNAs are not observed in isolated bacterial chromosomal or plasmid DNA samples after treatment with RNase A or I_f. The aggregate data suggest that they represent RNA fragments with nuclease-resistant secondary structures that may be unique to yeast.

Because downstream experiments often require accurate quantification of chromosomal DNA concentrations, we sought out methods that would yield pure, high quality DNA without contaminating RNAs. A series of trials using enzymatic, chemical and physical separation techniques were employed. These methods included use of multiple RNase enzymes incubated at various temperatures, selective destruction of RNAs by hydrolysis in strong base, preferential sedimentation of RNAs with lithium chloride, size-dependent precipitation using standard alcohol-based methods that combine NaOAc with ethanol or isopropanol, and chromatographic separation using anion exchange and gel chromatography spin columns. Using these approaches, we had only limited success removing the RNase-resistant fragments from the chromosomal DNA preparations.

A protocol published by Feliciello and Chinali [26], which involves addition of an isopropanol/potassium acetate solution to preferentially sediment larger nucleic acids while leaving smaller nucleic acids in the supernatant was also evaluated. The published protocol was unsuccessful in producing pure chromosomal DNA. We therefore sought to improve the method by testing several variables, including the volume and concentrations of alcohol and salt, centrifugation time, and reaction times. This testing process led to the development of a modified purification technique for chromosomal DNA minipreps that involves dilution of the isolated DNA, treatment with RNase A, incubation with 88% isopropanol/0.1 M KOAc, and centrifugation in a microcentrifuge. The DNA pellet is then washed with 70% ethanol, dried, and resuspended in the original volume of TE or water. The final optimized protocol is described in detail in the Methods. This method is rapid, yields high quality chromosomal DNA that lacks any detectable contaminating smaller fragments and exhibits improved A_{260}/A_{280} ratios that are consistent with pure DNA. Additionally, the purification results in little loss of the original chromosomal DNA, making it ideal for use in downstream experiments.

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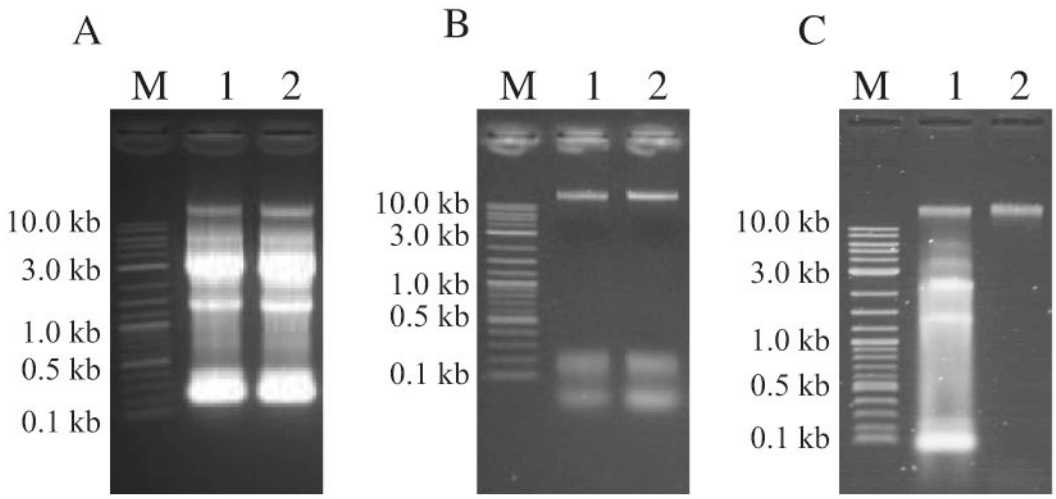

Figure 1.

Small RNase A-resistant bands are retained in yeast chromosomal DNA minipreps but not in *E. coli* bacterial chromosomal DNA minipreps. All DNA samples were run on 1.2% agarose gels. (A) Lanes 1 and 2, *S. cerevisiae* chromosomal DNA extracted using a simple SDS lysis method [22] without RNase treatment; two independent minipreps are shown; (B) Lanes 1 and 2, *S. cerevisiae* chromosomal DNAs prepared as in (A) and incubated with RNase A at 37°C consistently exhibit two resistant bands; (C) *E. coli* chromosomal DNA without RNase A treatment (lane 1) and after digestion with RNase A for 15 minutes at 37°C (lane 2). M, 2-log DNA ladder.

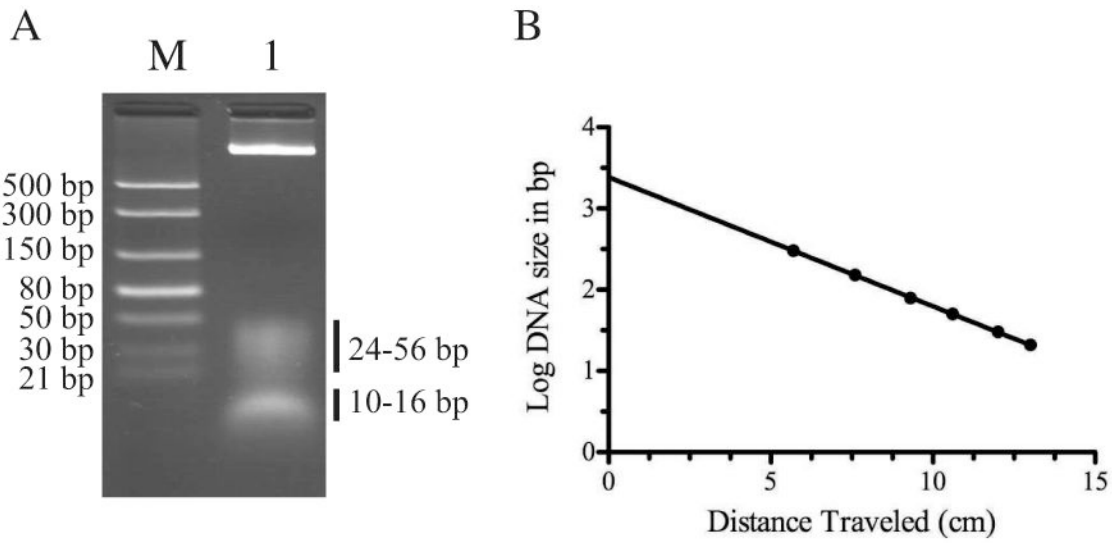

Figure 2.

The lower and upper RNase-resistant bands are composed of RNA fragments ranging in size from 10-16 bp and 24-56 bp, respectively. (A) Lane 1, *S. cerevisiae* chromosomal DNA treated with RNase A was run on a 3.5% agarose gel; M, dsRNA ladder; (B) The logs of the sizes of dsRNA ladder bands were plotted against their migration distances in order to determine the sizes of the RNAs within the RNase-resistant bands. The best fit trendline was calculated as $y=0.16x+3.4$ with $R^2=1.0$.

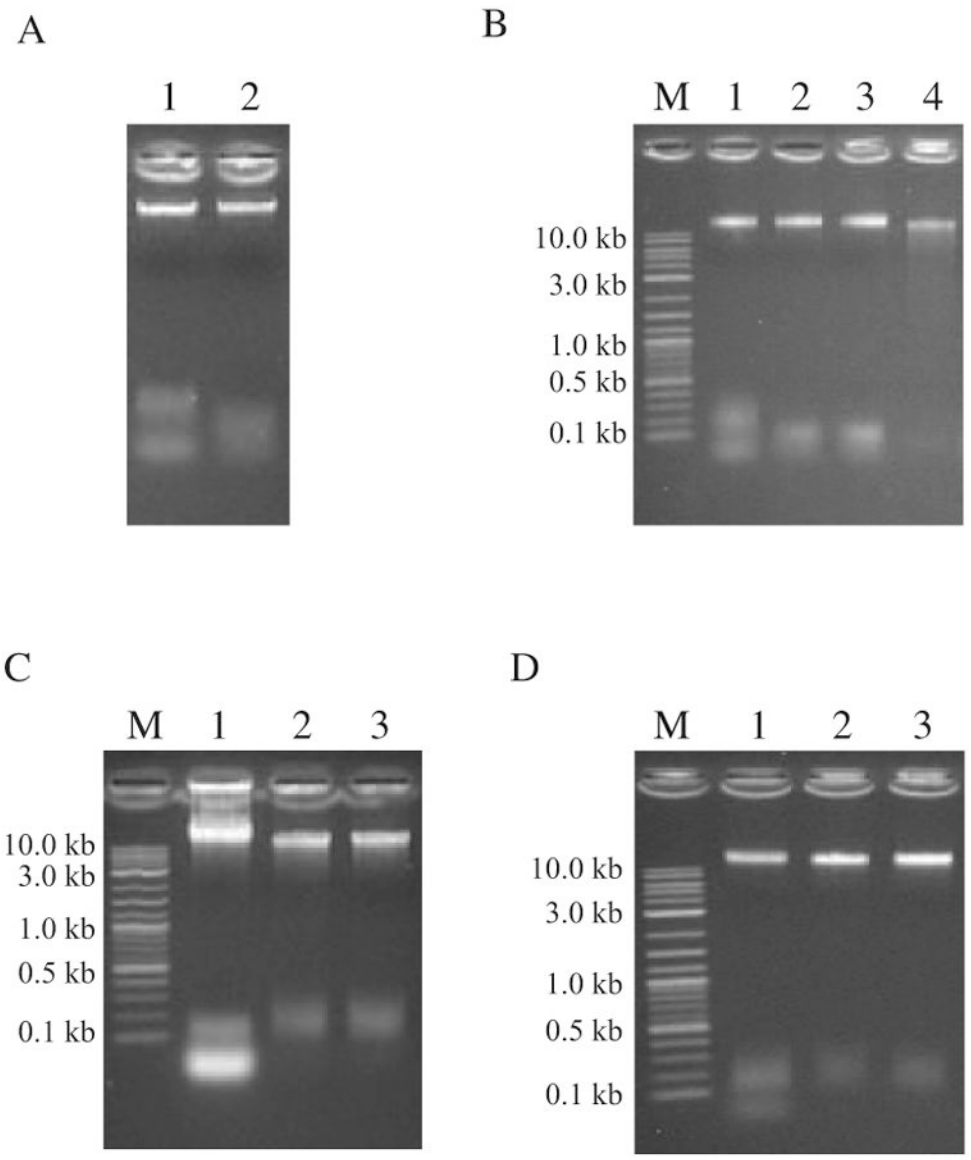

Figure 3.

Conventional physical and enzymatic methods were unsuccessful at removing both RNase-resistant populations from *S. cerevisiae* DNA minipreps. Samples were run on 1.2% agarose gels. Two bands persisted after treatment with RNase A (Lane 1 in panels A, B, C and D). (A) Lane 2, samples were heated to 65°C to reduce secondary structures prior to RNase A treatment; (B) Lanes 2 and 3, two independent DNA extractions were treated with RNase cocktail (a mixture of RNases A and T1); lane 4, the sample was treated with the dsRNA-specific nuclease RNase VI; (C) Lanes 2 and 3, DNA samples were treated with RNase A, then mixed with 5 volumes of PB buffer and purified using Qiagen anion exchange spin columns; (D) Lanes 2 and 3, RNase A-treated DNA samples were purified using CHROMA-1000 spin columns according to the manufacturer's instructions. M, 2-log DNA ladder.

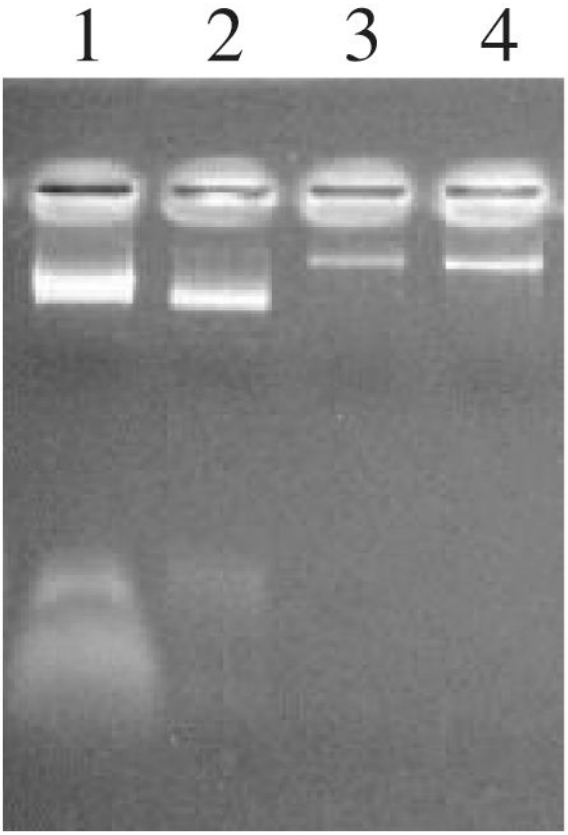

Figure 4.

Base hydrolysis methods eliminated the resistant RNA bands but strongly reduced the yield of chromosomal DNA. Lane 1, DNA after RNase A treatment; lane 2, DNA was digested with RNase A, followed by standard NaOAc/ethanol precipitation; lanes 3 and 4, DNAs were digested with RNase A, followed by treatment with either 0.2 M or 0.3 M NaOH. Similar results were observed with 0.2 M KOH.

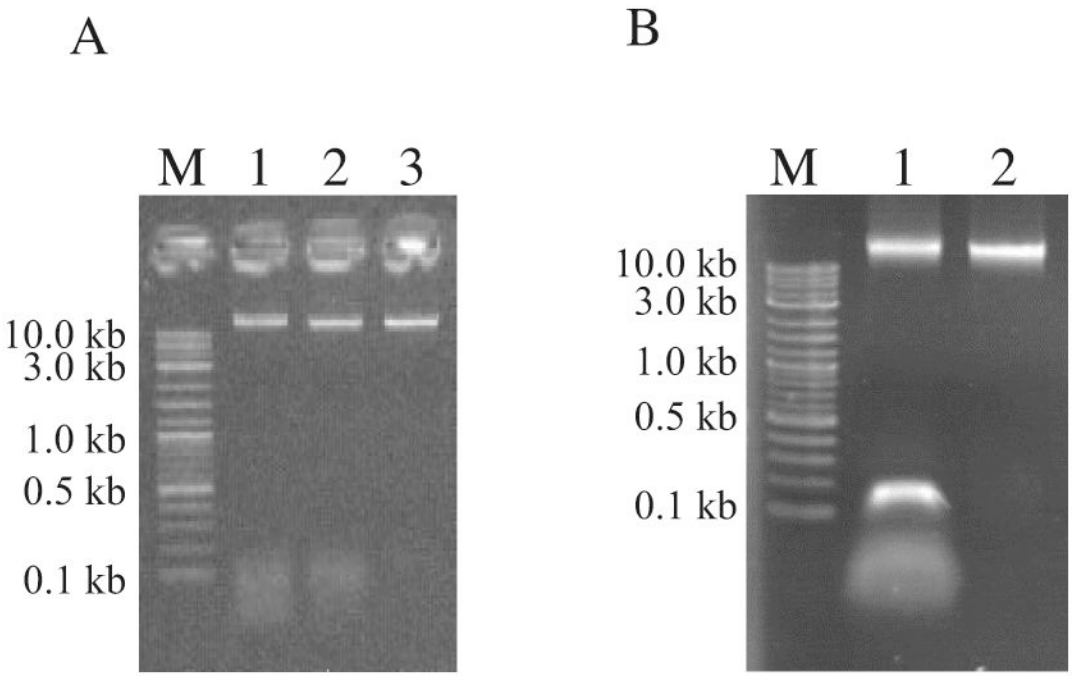

Figure 5.

Treatment of chromosomal DNA minipreps with potassium acetate and isopropanol removes the RNase-resistant RNA fragments. All samples were run on 1.2% agarose gels. (A) Representative examples of treatment with varying volumes of 88% isopropanol/0.2 M KOAc; lane 1, RNase A-treated DNA; lanes 2 and 3, representative experiment showing RNase A-treated DNA precipitated by addition of 1.2 and 0.6 volumes of 88% isopropanol/0.2 M KOAc, respectively. (B) Lane 1, miniprep DNA after RNase A treatment; lane 2, removal of all detectable RNAs by selective precipitation of chromosomal DNA with 0.6 volumes of 88% isopropanol/0.1 M KOAc using the optimized protocol. M, 2-log DNA ladder.