RNasin® Ribonuclease Inhibitor Part I: Characterization of the Protein

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Abstract

Ribonucleases (RNases) are ubiquitous enzymes that can detrimentally affect RNA isolation in the laboratory. Many applications such as RT-PCR and in vitro transcription and translation may be affected as well. RNase can renature after autoclaving and is difficult to inactivate. Many RNase inhibitors are available commercially. Promega's RNasin® Ribonuclease Inhibitor, one of the first introduced, is exceptionally effective at controlling RNase activity during molecular biology procedures.

Introduction

Placental ribonuclease inhibitor is a 50kDa protein that inhibits RNase by binding it in a 1:1 ratio with an association constant greater than $10^{16}M^{-1}$ (1). Placental ribonuclease inhibitor is expressed as a single-chain polypeptide consisting of 460 amino acid residues and contains leucine-rich repeats, a motif commonly associated with protein-protein interactions.

For applications such as RT-PCR, recombinant RNase inhibitors are advantageous because they are essentially free of contaminating eukaryotic DNA.

Promega's RNasin® Ribonuclease Inhibitor[a,b] is a noncompetitive inhibitor of RNases A, B and C, human placental RNase and angiogenin. Although RNasin® Ribonuclease Inhibitor inhibits a broad spectrum of RNases, it does not inhibit other nucleases, reverse transcriptase, or polymerases (Table 1). Additionally, Promega's RNasin® Ribonuclease Inhibitor is quality control tested for the absence of contaminating RNase activity including latent RNases, DNase activity and endonuclease activity. RNasin® Ribonuclease Inhibitor is available as the naturally occurring protein isolated from human placenta (Cat.# N2111) or as a recombinant protein expressed in *E. coli* (Cat.# N2511). For applications such as RT-PCR[c], recombinant RNase inhibitors are advantageous because they are essentially free of contaminating eukaryotic DNA.

Table 1. Effect of Natural and Recombinant RNasin® Ribonuclease Inhibitors on Selected Nucleases and Polymerases.

Inhibits	Does Not Inhibit
RNase A	RNase T1
RNase B	S1 Nuclease
RNase C	RNase from *Aspergillus*
human placental RNase	RNase H
angiogenin	RNase ONE™ Ribonuclease
	Taq DNA Polymerase
	AMV or M-MLV Reverse Transcriptase
	SP6, T7, T3 RNA Polymerase

Characterization of RNasin® Ribonuclease Inhibitor

Total Yeast RNA Assay for the Measurement of RNase Activity: In this article we present data that describes the ability of RNasin® Ribonuclease Inhibitor to protect total yeast RNA from degradation in the presence of RNase A using a total yeast RNA assay. The standard RNase activity assay (the cCMP hydrolysis assay), uses an artificial substrate for RNase (cCMP) and therefore might not directly correlate with actual RNA degradation. However, the total yeast RNA assay used in these studies allows the characterization of RNasin® Ribonuclease Inhibitor under reaction conditions that more closely resemble standard laboratory procedures. In the total yeast RNA assay, RNA is incubated alone or with RNase in an assay mix that contains an appropriate buffer and 5mM magnesium in a total volume of 0.5ml. After incubations, 0.5ml 10% TCA is added to stop the reaction and to precipitate large RNA species. The addition of small amounts of RNase A to >20µg (40µg/ml) RNA followed by incubation at 37°C for 15 minutes results in degradation of RNA into TCA-soluble material that can be quantitated by spectrophotometric analysis at OD_{280} (Figure 1). The amount of ribonucleotides or small RNA fragments in the supernatant following TCA precipitation is a measure of the RNase activity present.

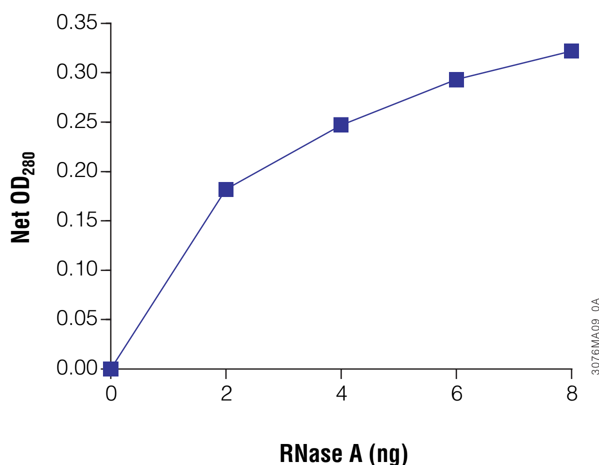

Figure 1. Total yeast RNA assay for RNase Activity. Total yeast RNA was incubated in the presence of increasing concentrations of RNase A for 15 minutes at 37°C in 0.5ml of reaction mix containing 50mM MOPS and 5mM $MgCl_2$ (pH 6.5). After a 15-minute incubation, 0.5ml 10% TCA was added to stop the reaction and precipitate the large RNA molecules. An OD_{280} measurement was taken of the TCA-soluble material.

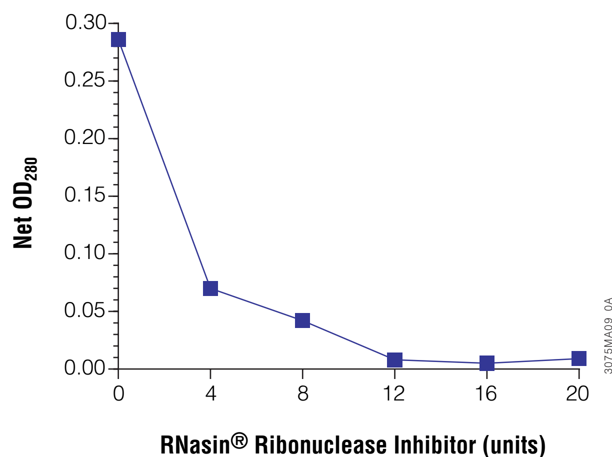

Figure 2. Total yeast RNA assay showing inhibition of RNase A by Promega's RNasin® Ribonuclease Inhibitor. Total yeast RNA was incubated in the presence of 5ng RNase A at 37°C for 15 minutes in 0.5ml of reaction mix containing 50mM MOPS, 5mM $MgCl_2$ (pH 6.5). After incubation, 0.5ml of 10% TCA was added to stop the reaction and precipitate large RNA molecules. An OD_{280} measurement was made of the TCA soluble material. Experimental reactions were compared to a RNase/no inhibitor control to determine the percent inhibition of RNase A activity.

We used the assay described above to characterize the ability of RNasin® Ribonuclease Inhibitor to prevent RNA degradation by RNase A. Total yeast RNA was incubated alone, with 5ng RNase, or with 5ng RNase plus RNasin® Ribonuclease Inhibitor for 15 minutes at 37°C. The reaction was stopped and RNA precipitated as described above. We compared the RNase activity in the experimental reaction (RNA, RNase, inhibitor) to the positive control for RNase activity (RNA, RNase, no inhibitor) for each trial to determine inhibition of RNase activity by the ribonuclease inhibitor. As expected in the RNase positive control reaction, the RNA was degraded and detectable in the TCA-soluble material. For the experimental reaction, we incubated total yeast RNA with 5ng RNase and RNasin® Ribonuclease Inhibitor at a variety of concentrations at 37°C for 15 minutes. For this assay, the RNase A and the RNase inhibitor were added simultaneously. The RNasin® Ribonuclease Inhibitor clearly protected RNA from degradation in this assay (Figure 2).

RNasin® Ribonuclease Inhibitor protects RNA from degradation

Temperature (4–50°C): To demonstrate that RNasin® Ribonuclease Inhibitor does not lose activity outside typical physiological conditions, we performed experiments showing that the inhibitor successfully protects RNA from degradation under diverse reaction conditions. We tested the ability of RNasin® Ribonuclease Inhibitor to inhibit RNase at 4, 25, 37 and 50°C in the total yeast RNA assay. The ratio of RNase to RNasin® Ribonuclease Inhibitor (5ng:8 units) was kept constant at each temperature. RNasin® Ribonuclease Inhibitor blocked nearly 100% of RNase A activity at each of the temperatures assayed.

pH Value (pH 5.5–9.0): We also tested RNasin® Ribonuclease Inhibitor to determine the pH range at which it most effectively inhibits RNase activity. Buffers containing no DTT were made, ranging from pH 5.5 to 9.0. Total yeast RNA was incubated in the presence of 5ng RNase and 8 units RNasin® Ribonuclease Inhibitor. At all pH values tested, RNasin® Ribonuclease Inhibitor effectively prevented RNA degradation. Figure 3 clearly demonstrates that RNasin® Ribonuclease Inhibitor is effective over a wide range of pH values.

Absence of Reducing Conditions: Promega's technical literature for RNasin® Ribonuclease Inhibitor indicates that it is best used in reactions where a reducing agent such as DTT is present. All proteins, including ribonuclease inhibitors, can be adversely affected by oxidation over an extended time period. We recommend that a reducing agent be used with RNasin® Ribonuclease Inhibitor to ensure its ability to protect important RNA samples from degradation *over extended periods of time*. However, high levels of reducing agent, such as 1mM DTT, are not required for the activity of RNasin® Ribonuclease Inhibitor.

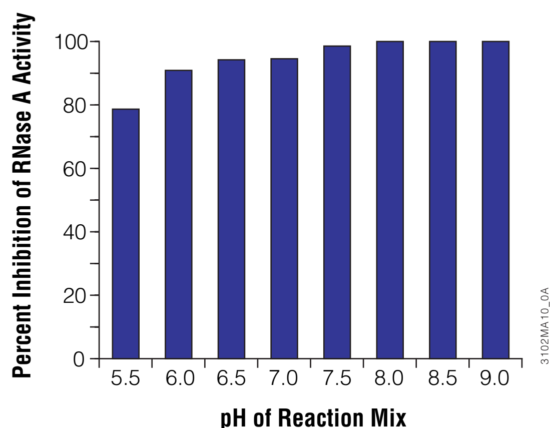

Figure 3. Ability of Promega's RNasin® Ribonuclease Inhibitor to inactivate RNase A at various pH. Total yeast RNA was incubated in the presence of 5ng RNAse and 8 units RNasin® Ribonuclease Inhibitor. The activity of RNase A in the presence of RNasin was compared to the activity of RNase A alone at each pH.

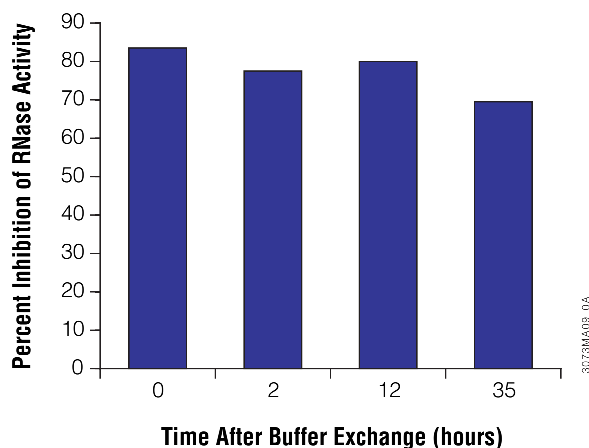

Figure 4. Inhibition of RNase A by Promega's RNasin® Ribonuclease Inhibitor in the absence of reducing conditions. RNasin® Ribonuclease Inhibitor was diluted 1:8 and fractionated through a Centri-Sep™ cartridge. The protein was further diluted 1:10 and stored at 4°C (0.5 unit/µl RNasin® Ribonuclease Inhibitor final concentration). Ability to inhibit RNase A was assayed over time using the total yeast RNA assay.

We have assayed RNasin® Ribonuclease Inhibitor under conditions in which we have attempted to remove all reducing agent. In one such study, RNasin® Ribonuclease Inhibitor was diluted 8-fold and fractionated through a Centri-Sep™ cartridge to remove small molecules from the protein. Next, we further diluted RNasin® Ribonuclease Inhibitor 1:10 to obtain a solution of the protein that was essentially free of reducing agent. We produced and stored this solution in a normal atmosphere; therefore it was exposed to atmospheric oxygen for up to 35 hours. The activity of the RNasin® Ribonuclease Inhibitor was assayed immediately and over time at 4°C. RNasin® Ribonuclease Inhibitor successfully inhibited RNase A after 35 hours at 4°C (Figure 4). These results clearly indicate that the presence of reducing agent is not essential for inhibition of RNase activity by RNasin® Ribonuclease Inhibitor over the time frames typical of laboratory applications.

RNasin® Ribonuclease Inhibitor blocked nearly 100% of RNase A activity at each of the temperatures assayed.

Quality assurance assays for RNasin® Ribonuclease Inhibitor

Each lot of RNasin® Ribonuclease Inhibitor is subjected to stringent quality control testing. Promega's RNasin® Ribonuclease Inhibitor is tested for the presence of contaminating RNase activity in two ways. One microgram of RNA is incubated with 200 units of RNasin® Ribonuclease Inhibitor for 1 hour at 37°C, and the RNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of degradation. To test for latent RNase activity, RNasin® Ribonuclease Inhibitor is heat-denatured at 67°C for 15 minutes, and the equivalent of 200 units are then incubated with 1µg of RNA for 1 hour at 37°C. In addition to testing for contaminating RNase activity, RNasin® Ribonuclease Inhibitor is tested for DNase activity and endonuclease activity. For additional information about quality control assays, see the Promega Product Information (Part# 9PIN211), available by request from Promega or online at www.promega.com.

Conclusions

RNasin® Ribonuclease Inhibitor functions robustly at a variety of temperatures and pH, allowing the researcher flexibility in designing experiments. RNasin® Ribonuclease Inhibitor can inhibit RNase in the presence or absence of DTT, and because it is not isolated from mammalian tissues or sera, Recombinant RNasin® Ribonuclease Inhibitor reduces the risk of contamination with mammalian DNA or RNA, a significant advantage in applications such as RT-PCR, where even low levels of nucleic acid contaminants can result in amplification of unwanted products. RNasin® Ribonuclease Inhibitor works well in highly sensitive techniques such as single-cell RT-PCR, in vitro RNA synthesis and in vitro translation.

Reference

1. Lee, F.S. *et al.* (1988) *Biochemistry* **27**, 8545–8553.

Protocols

- *RNasin® Ribonuclease Inhibitor Product Information* #9PIN211, Promega Corporation. (www.promega.com/tbs/9pin211/n211abst.html)
- *Recombinant RNasin® Ribonuclease Inhibitor, Product Information* #9PIN251, Promega Corporation. (www.promega.com/tbs/9pin251/n251abst.html)

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Ordering Information

Product	Size	Cat.#
RNasin® Ribonuclease Inhibitor[a]	2,500 units	N2111
	10,000 units	N2115
Recombinant RNasin® Ribonuclease Inhibitor[a,b]	2,500 units	N2511
	10,000 units	N2515

RNasin is a trademark of Promega Corporation and is registered with the U.S. Patent and Trademark Office. RNase ONE is a trademark of Promega Corporation.
Centri-Sep is a trademark of Princeton Separations, Inc. SUPERase•In is a trademark of Ambion, Inc.

[a]U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

[b]U.S. Pat. No. 5,552,302.

[c]The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support unauthorized or unlicensed use of the PCR process.

Product Bibiolgraphy

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