

RNase Protection Assay System: A Versatile Technique for the Analysis of RNA

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Promega's RNase Protection Assay System combines the unique properties of RNase ONE(TM) Ribonuclease into a procedure that provides more reproducibility than methods employing other RNases or S1 nuclease. With simple modifications, the system also can be used to detect single base pair mismatches.

Introduction

The ribonuclease protection assay is an extremely sensitive technique for the quantitation of specific RNAs in solution (1-3). The ribonuclease protection assay can be performed on total cellular RNA or poly(A)-selected mRNA as a target. The sensitivity of the ribonuclease protection assay derives from the use of a complementary *in vitro* transcript probe which is radiolabeled to high specific activity. The probe and target RNA are hybridized in solution, after which the mixture is diluted and treated with ribonuclease (RNase) to degrade all remaining single-stranded RNA. The hybridized portion of the probe will be protected from digestion and can be visualized via electrophoresis of the mixture on a denaturing polyacrylamide gel followed by autoradiography. Since the protected fragments are analyzed by high resolution polyacrylamide gel electrophoresis, the ribonuclease protection assay can be employed to accurately map mRNA features such as initiation and termination sites (1,4,5). If the probe is hybridized at a molar excess with respect to the target RNA, then the resulting signal will be directly proportional to the amount of complementary RNA in the sample.

The ribonuclease protection assay is superior to Northern blots for the detection and quantitation of low abundance RNAs for numerous reasons. With Northern blots, RNA transfer and binding to the membrane may be inefficient. Once bound, some RNA molecules may not be accessible for hybridization. Finally, sample integrity influences the degree to which the signal is localized in a single band (6). Also, improvements in the RNase Protection Assay System have overcome the shortcomings of the original S1 nuclease protection assay because with S1 mapping procedures, lower temperatures are used to favor single-stranded cleavage, AU-rich regions are attacked non-specifically and there are nibbling artifacts (2,7).

Promega's RNase Protection Assay System utilizes RNase ONE(TM) (8). RNase ONE(TM) is one of the few known ribonucleases that shows no bias, cleaving the phosphodiester bond between any two ribonucleotides and degrading single-stranded RNA to a mixture of monomers and oligomers (9,10). In addition, RNase ONE(TM) is inactivated by 0.1% sodium dodecyl sulfate (SDS) (11), eliminating the need for organic extractions to remove the RNase before gel analysis. Elimination of organic extractions vastly improves the reproducibility and accuracy of the assay. This is in contrast to ribonuclease protection assays that employ RNase A, RNase P1, RNase T1, or S1 nuclease, which require phenol:chloroform extraction so the protected fragments are not degraded upon electrophoresis. The Promega RNase Protection Assay System represents a significant improvement in the standard RNase Protection Assay method and incorporates the principles of more recently described rapid techniques

(12,13).

Standard Rnase protection assay

To demonstrate the effectiveness of Promega's RNase Protection Assay System, beta-actin messages were mapped using differing amounts of murine liver RNA as a target. The beta-actin probe was generated *in vitro* and was approximately 350 bases in length. [Figure 1](#) presents these results, demonstrating the appropriately sized protected fragment and the level of sensitivity for this experiment.

With the RNase Protection Assay System, RNA sample purity is critical for accurate results. The target RNA should contain no contaminating proteins. In addition, samples should be free of any traces of guanidine thiocyanate, which will inhibit the activity of RNase ONE(TM).

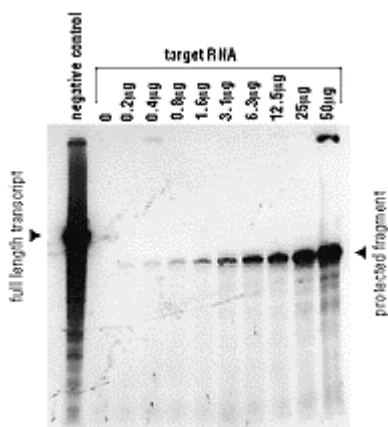


Figure 1. Titration of target RNA for use in the RNase Protection Assay. An RNase protection assay was performed using various amounts (0-50µg) of Control RNA as the target RNA. The assay was performed as described in the manual supplied with the system (14). Radiolabeled probe RNA was generated by *in vitro* transcription using the Actin Control Plasmid and SP6 RNA Polymerase. The probe was gel-purified and 150,000cpm were used in each reaction. Hybridizations were performed at 50°C for 2 hours. Digestion was performed with 10 units of RNase ONE(TM) at 22°C (room temperature) for 1 hour. Samples were separated on a 5% polyacrylamide gel. Following electrophoresis, the gel was dried and exposed to X-ray film for 16 hours at room temperature.

Single-base mismatch analysis

RNase analysis of single base pair mismatches was demonstrated by using radiolabeled negative strand complementary transcripts of the wild-type beetle luciferase gene hybridized to several different mutant target transcripts. All transcripts were generated *in vitro* from purified, linear plasmid templates. The mismatches were selected to investigate a small region of the gene to minimize the contribution of various secondary structure effects to the results. Table 1 lists the mismatches tested.

Table 1. Single Base Mismatches Tested in Promega's RNase Protection Assay System.

Mutant	Change	Resulting RNA Mismatch (Template/radiolabeled probe)
I248#2	T-->C	A/C

S247#5	C-->T	G/U
S247#6	C-->A	G/A
S247#7	T-->A	A/A

There are three general categories of mismatches which can be tested: purine/purine (Pu/Pu), purine/pyrimidine (Pu/Py) and pyrimidine/pyrimidine (Py/Py). In this experiment, Pu/Pu and Pu/Py mismatches were tested, in particular the G/U pseudomismatch and mismatches involving adenine. These latter are significant because RNase A cleaves after C and U residues and RNase T1 cleaves after guanine, but neither cleaves after adenine. Adenine was also placed on each of the two strands in the mismatch to see if there was polarity to the cleavage using RNase ONE(TM). The other possible mismatches: G/G, C/C, U/U, and U/C were not investigated simply because they were not available in the region of the gene under investigation.

For each reaction, 5fmol of a 416 base ³²P-labeled probe (approximately 50,000cpm) and 1pmol of a specific target transcript were annealed in the hybridization reaction and processed using the standard RNase Protection Assay protocol except the number of units of RNase ONE(TM) was varied to determine the optimum level for mismatch detection.

The results from this analysis indicated that RNase ONE(TM) recognized all but the G/U pseudomismatch. In the case of the pseudomismatch, the results were indistinguishable from those obtained for the fully matched wild-type complements (data not shown). **Figure 2** provides an example of the A:A mismatch at serine 247. In this particular example, the expected full-length protected fragment is 344 bases. If the mismatch is recognized by RNase ONE(TM), then fragments of 221 and 123 bases are expected. The results show that the mutant-wild type difference was detected optimally with 30 units of RNase ONE(TM). For detection of single base mismatches, we recommend using approximately 100 times more RNase ONE(TM) than is normally used in the standard protection assay.

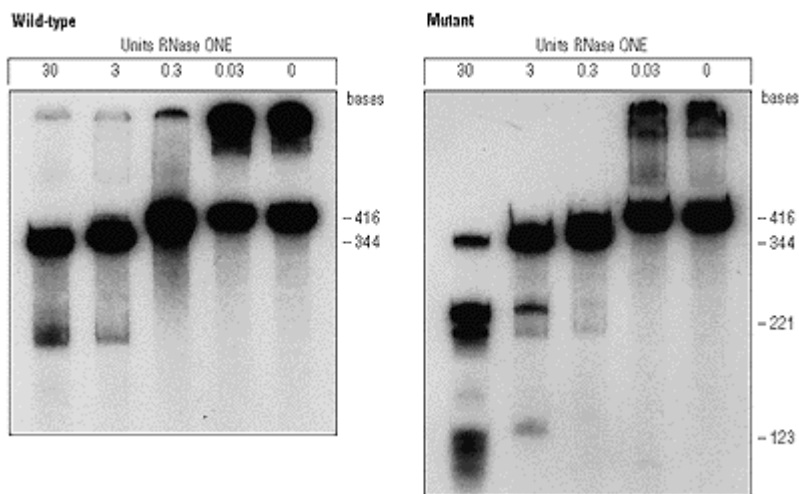


Figure 2. Detection of a single base mismatch at serine 247 of the luciferase gene. The RNase Protection Assay System was used to detect a single base mismatch between a radiolabeled probe transcript of the wild-type beetle luciferase gene and a mutant target transcript. All of the transcripts were synthesized *in vitro* from linear plasmid templates. The A:A mismatch is located within the codon for serine 247. The assay was performed using 5fmol (687pg) of probe transcript and 1pmol (330ng) of target transcript. Hybridization was performed at

42°C for 30 minutes. Hybrids were digested with RNase ONE(TM) (0-30 units) for 50 minutes at 22°C (room temperature). Results of the assay were visualized by autoradiography following electrophoresis on a 4.5% polyacrylamide gel. The length of the target and probe transcripts are 1000 bases and 416 bases, respectively. The length of the fully protected fragment is 344 bases.

Summary

The Promega RNase Protection Assay System provides the reagents necessary to map RNAs more accurately than obtained with Northern blot procedures. In addition, the system uses RNase ONE (TM) which shows no bias in cleavage compared to other RNases or S1 Nuclease used in other protection procedures. Finally, Promega's RNase Protection Assay System can be used to perform single base pair mismatch analysis, an important feature when comparing mutants or variants of genes.

References

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Ordering Information

Product	Cat.#
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RNase Protection Assay System	F1520
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This system contains sufficient reagents to perform 100 RNase protection assays using up to 10µg total RNA or 1µg mRNA per assay.

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