

Isolation and Purification of Ribosome-Inactivating Proteins

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Summary

Ribosome-inactivating proteins (RIPs) are cytotoxic *N*-glycosidases identified in plants, fungi, and bacteria. RIPs inhibit protein synthesis by virtue of their enzymatic activity, selectively cleaving a specific adenine residue from a highly conserved, surface-exposed, stem-loop (S/R loop) structure in the 28S rRNA of ribosomes. Some RIPs also exhibit a number of other enzymatic activities such as RNase, DNase, phospholipase, and superoxide dismutase (SOD). RIPs are considered to be plant defense-related proteins as they are able to inhibit the multiplication and growth of several pathogenic virus, fungi, and bacteria either alone or in conjugation with other defense-related proteins. The mechanism of inhibitory activity of RIPs against fungal pathogens seems to be by directly inhibiting fungal growth rather than depurinating host plant ribosomes and causing cell death as previously envisaged. This chapter describes the protocol used to isolate and purify RIPs from plant tissues.

Key Words: Antimicrobial activity; depurination; plant defense; ribosome-inactivating protein.

1. Introduction

Ribosome-inactivating proteins (RIPs) are cytotoxic *N*-glycosidases identified in plants, fungi and bacteria (**Table 1**). RIPs are largely divided into two classes: (1) type I RIPs consist of a single *N*-glycosidase domain; and (2) type II RIPs are chimero-RIPs constructed of an A-chain, functionally equivalent to a type-I RIP, which is attached to a sugar-binding B-chain lectin domain (**I**). RIPs have been proposed to inhibit protein synthesis by virtue of their enzymatic activity, selectively cleaving an adenine residue from a highly conserved,

¹The first two authors contributed equally to this work.

Table 1
List of Ribosome-Inactivating Proteins

Plant	Source	Name of RIP
Type I		
<i>Phytolacca americana</i>	Leaves	PAP
	Leaves (seasonally expressed)	PAP-I/II/III
	Seeds	PAP-S
	Roots	PAP-R
<i>Phytolacca insularis</i>		PIP
		PIP2
<i>Phytolacca dioica</i>	Seeds	PD-S2
	Leaves	PD-L1/2/3/4
<i>Phytolacca dodecandra</i>	Leaves	Dodecandrin
<i>Mirabilis expansa</i>	Roots	ME 1/2
<i>Mirabilis jalapa</i>	Leaves	MAP
<i>Gelonium multiflorum</i>	Seeds	Gelonin
<i>Hura crepitans</i>	Latex	RIP
<i>Manihot palmata</i>	Seeds	Mapalmin
<i>Bryonia dioica</i>	Leaves	Bryodin-L
	Roots	Bryodin
<i>Citrullus colocynthis</i>	Seeds	Colocin 1/2
<i>Luffa cylindrica</i>	Seeds	Luffin a
		Luffin b
<i>Momordica charantia</i>	Seeds	Momordin
<i>Momordica cochinchinensis</i>	Seeds	Momorcochin
<i>Trichosanthes kirilowii</i>	Roots	Trichosanthin
		TAP-29
	Seeds	Trichokirin
<i>Hordeum vulgare</i>	Seeds	barley RIP
<i>Triticum aestivum</i>	Germ	Tritin
<i>Zea mays</i>	Seeds	Corn RIP
<i>Asparagus officinalis</i>	Seeds	Asparin 1/2
<i>Dianthus caryophyllus</i>	Leaves	Dianthin 30/32
<i>Lychnis chalconica</i>	Seeds	Lychnin
<i>Saponaria officianlis</i>	Seeds	Saporin 5/6/9
<i>Cinnamomin camphora</i>		Cinnamomin
		Camphrin
<i>Trichosanthes anguina</i>	Seeds	Trichoanguin
<i>Iris hollandica</i>	Bulbs	Iris RIP.A1/2/3
<i>Volvariella volvacea</i>	Fruiting Bodies	Volvarin
<i>Sechium edule</i>	Seeds	Sechiumin
<i>Hypsizigus marmoreus</i>	Fruiting bodies	Hypsin

(continued)

Table 1 (continued)
List of Ribosome-Inactivating Proteins

Plant	Source	Name of RIP
Type I		
<i>Lyophyllum shimeji</i>	Fruiting Bodies	Lyophyllin
<i>Cucurbita pepo</i>	Fruits	Pepocin
<i>Sambucus nigra</i>	Fruits	Nigritin
Type II		
<i>Ricinus communis</i>		Ricin
<i>Abrus precatorius</i>		Abrin
		Abrin II
<i>Sambucus nigra</i>	Bark	Nigrin b
		Basic Nigrin b
<i>Sambucus sieboldiana</i>	Bark	Sieboldin-b
<i>Sambucus ebulus</i>	Leaves	Ebulin 1
<i>Momordica charantia</i>	Seeds	B-momorcharin
<i>Iris hollandica</i>	Bulbs	Agglutinin b/r
Type III		
<i>Hordeum vulgare</i>	Leaves	JIP60
<i>Zea mays</i>	Seeds	Maize RIP

surface-exposed, stem-loop (S/R loop) structure in the 28S rRNA (2). Thus, the site-specific deadenylation interrupts the interaction of elongation factors, EF1 and EF2, with the S/R loop, and blocks protein synthesis at the translocation step. Recently, Type-III RIPs have been identified as a single chain containing an extended carboxyl-terminal domain with unknown function; these are synthesized as precursors requiring proteolytic removal of an internal peptide for activity (3).

1.1. Enzymatic Activity

RIPs are presently classified as rRNA *N*-glycosidases in the enzyme nomenclature (EC 3.2.2.22). Several studies have suggested that RIPs specifically cleave the *N*-C glycosidic bond of the adenine base in the tetra loop sequence (GAGA) located on the sarcin/ricin (S/R) loop of eukaryotic and prokaryotic ribosomes. The universally conserved adenine residue A4324 of the eukaryotic 28S rRNA (and A2660 in the prokaryotic 23S rRNA) has long been considered the only enzymatic target site for RIPs (2,4), but several lines of evidence have recently revealed alternative substrates to the S/R loop. For instance, it has been shown that several RIPs can release adenine from multiple

sites in rRNA (5). Furthermore, RIPs have been found to target various nucleic acids, randomly removing adenine residues from single-stranded regions of nucleic acids and, to a lesser extent, guanine residues from wobble base-pairs in hairpin stems. This substrate recognition and enzymatic activity depends on the physical availability of nucleotides; denaturation of nucleic acid structures increases their interaction with RIPs (5,6).

Apart from adenine and guanine glycosidase activity, RIPs exhibit a number of novel enzymatic activities such as RNase, DNase, phospholipase, and superoxide dismutase (SOD) activities (7). These observations suggest that RIPs may possess dual biochemical activities and multiple biological roles, renewing our interest in the biological functions of RIPs because of the potential for diverse functions *in planta* of the possible primary/secondary roles of RIPs.

1.2. Antimicrobial Activity

RIPs are considered to be defense-related proteins *in planta* as RIPs are able to inhibit the multiplication and growth of several pathogenic organisms including virus, fungi, and bacteria. Despite differences in virus infection mechanisms, a number of RIPs show broad-spectrum antiviral activity against both plant and animal viruses including human immunodeficiency virus (HIV). In addition, several RIPs have shown direct inhibitory activity against the growth of various fungal and bacterial pathogens such as *Alternaria solani*, *Alternaria alternaria*, *Agrobacterium tumerfaciens*, *Agrobacterium rhizogenes* R100nal, *Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium proliferatum*, *Mycosphaerella arachidicola*, *Neurospora crassa*, *Phycomyces blakesleeanus*, *Pythium irregulare*, *Physalospora piricola*, *Trichoderma reesei*, and *Verticillium dahliae*, *Bacillus subtilis*, *Rhizobium leguminosarum*, *Serratia marcescens*, *Pseudomonas syringae*, *Xanthomonas campestris* pv *versicatoria*, and *Erwinia carotovora* (1).

Some RIPs, interestingly, show synergistic antifungal effects combined with other plant defense-related proteins (8,9). The recently isolated root-specific PAP-H also shows traces of inhibitory activity against several fungal pathogens in the presence of other pathogenesis-related (PR) proteins such as chitinase and β -1,3-glucanase (10). Facilitating this possible synergistic effect, disease resistance in transgenic tobacco plants is manifested by enhanced antifungal activity when the barley RIP is constitutively co-expressed with barley class II chitinase (11). The combination of barley endosperm RIP with barley class II chitinase produces a significant increase in resistance to *Rhizotonia solani* in transgenic tobacco. Both transgenic proteins (RIP + chitinase) accumulate in the intercellular spaces of transgenic tobacco, and it was postulated that the cytotoxic effect of the barley RIP on fungal cells is enhanced by

chitinase action resulting in an increased amount of RIP entering fungal cells. Maize RIP, b-32, under control of the potato *wun1* gene promoter, also increases the tolerance of transgenic tobacco to *R. solani* (12).

1.3. Classic Theory of Antimicrobial Mechanism

The defense mechanism of RIPs was explained previously by the so-called “suicide model” similar to the hypersensitive response (HR) (13). The inhibitory mechanism of RIPs against microbial pathogens has been postulated based on the evidence and mechanistic information available from PAP and its isoform (10). Based on the RI activity and the extracellular localization of PAP, it has been suggested that RIPs are synthesized in an inactive form, sequestered in the cell wall matrix, and re-enter the cytoplasm along with the pathogen at the infection sites. Thus, RIPs inhibit pathogen multiplication by inactivating host ribosomes and causing host cell death. However, the antimicrobial activity and mechanism of RIPs are gaining new attention through recent studies.

1.4. Revising the Working Model

As discussed, the widely accepted mechanism for antimicrobial action identifies host ribosomes as the target of RIP activity. However, no evidence has been discovered that would prove this suicidal mechanism. Several experiments with transgenic plants expressing RIPs also do not exhibit HR or other symptoms of spontaneous cell death in response to microbial infection although they are resistant to a wide range of pathogens. In addition, no pathogen has been reported to evoke the suicidal machinery in any plant system producing RIP(s).

Several independent studies suggest that the antimicrobial activity of RIPs can be independent from the enzymatic activity of RIPs, inactivating ribosomes of the plant host. The earliest demonstrations were made by Tumer and her colleagues, who assayed deletion mutants of PAP in transgenic tobacco plants (9,13,14). Although PAPc (PAPW327stop) and PAPn (PAPG75D) were found to be unable to depurinate the S/R loop, they still conferred resistance against plant viral and fungal pathogens. Further experiments revealed that PAP is able to directly depurinate selective viral mRNA that has a 5' terminal m7GpppG cap; in contrast, PAP has no significant effect on uncapped mRNAs (15,16). From these observations, the authors hypothesized that during viral infection, PAP may target selective viral RNAs by binding to the cap instead of inactivating host ribosomes. Recently, the inhibitory mechanism of RIPs against HIV replication that has generally been believed to relate to RI activity has also been readdressed (17). Wang et al. (17) use site-directed mutagenesis approaches with TCS to demonstrate that the classical RI activity of TCS is not adequate to explain the anti-HIV action of TCS. In this study, an exception is

revealed: TCS mutants with a C-terminal deletion or addition of amino acids retained almost all RI activity, but were devoid of anti-HIV activity. This result demonstrates that the C-terminus region of TCS is responsible for anti-HIV activity, and suggests that the defensive mechanism of RIPs against pathogens can be separated from RI activity.

As for the inhibitory mechanism of RIPs against fungal pathogens, more and more evidence has shown that RIPs can directly target and inhibit fungal growth rather than depurinating host plant ribosomes and causing cell death. The type-I RIPs—ME1, hypsin, lyophyllin, and hispin (*Benincasa hispida*)—show a direct inhibitory activity against an array of pathogenic and nonpathogenic fungi (8–10,18,19). This fungal cytotoxicity has also been shown to be independent from RI activity (20). Comparing three different RIPs—ME1, RTA, and saporin-S6 (isoform of saporin)—the latter two showed approx 10- to 50-fold higher enzymatic activity against isolated fungal ribosomes than did ME1. Nevertheless, ME1 showed higher inhibitory activity against fungal growth itself compared to RTA and saporin-S6. A study with transgenic tobacco plants expressing PAP and its mutants also demonstrates that the RI activity of RIP is not sufficient for host resistance to the fungal pathogen (14). To further understand the antifungal mechanism of RIPs, Park et al. (20) labeled ME1 and saporin-S6 with NHS-fluorescein and the interaction between labeled RIPs and fungal cells was monitored. The labeled ME1 showed strong interaction with fungal hyphae, translocated to the cell wall, and presumably penetrated into the cytosolic region, in contrast to the absence of interaction observed between labeled saporin-S6 and fungi. This result indicated that some type-I RIPs, lacking any known carbohydrate-binding domain, are capable of interacting with the target cell surface. Taken together, these results indicate that: (1) the RI activity of RIPs does not adequately explain their defensive action against pathogens; (2) the defensive action of RIPs is independent from their enzymatic specificity; (3) some RIPs are able to recognize and target selective pathogens by possibly binding to specific cell membrane components; and, finally, (4) the defense mechanism of RIPs could proceed by directly targeting invading pathogens rather than host ribosomes.

1.5. Therapeutic Properties

The target specificity of monoclonal antibodies induced the development of immunotoxins, which deliver toxins, such as RIPs, to specific cells. PAP linked to specific antibodies has been shown to prevent the growth of leukemia cells (21). Human CD19+ mixed lineage leukemia cells (RS4-11) proliferate in the hematopoietic tissues and other organs of mice with severe combined immunodeficiency in a manner similar to human acute leukemia. PAP linked to spe-

cific antibodies (anti-CD19-Pokeweed antiviral protein immunotoxin) selectively inhibit clonogenic RS4,11 cells *in vitro*. This immunotoxin is thus able to extend the life span of the mice inoculated with RS4,11 cells (22).

Another relatively successful immunotoxin is TP3-PAP. This antibody (TP3) is reactive against an antigen on human and canine osteosarcoma. This tumor-associated antigen is expressed at very high levels on the surface membrane of human osteosarcoma cells (23), and the antibody (IgG2b anti-P80, TP3) reacts with mesenchymal tumors like osteosarcomas, hemangiopericytomas, chondrosarcomas, malignant fibrous histiocytomas, and synovial cell sarcomas (23). Studies with TP3 attached to PAP (TP3-PAP) show high activity against osteosarcoma cells *in vitro*, killing all the culture cells after 48 h.

Finally, studies with immunotoxins prepared by linking momordin 1, pokeweed antiviral protein from seeds (PAP-S), and saporin-S6 to the monoclonal antibodies 48-127, which recognize a glycoprotein (gp54) expressed in human bladder carcinomas, have shown that RIPs linked to those antibodies are effective against bladder tumor cell lines (T24) (24). The same three RIPs linked to Ber-H2 monoclonal antibodies directed against CD30 antigen of human lymphocytes induced apoptosis in CD30 cell lines (25). Furthermore, RIP gelanin was also shown to be effective on malarial parasites when linked to human transferrin (26). These studies indicate that RIPs could be very effective drugs when linked to proper targeting antibodies, and could thus be used as antitumor drugs.

1.6. Future Perspectives

Although RIPs have been studied for many years and their enzymatic and biological activities have been extensively investigated, very little is known about their role in plant biology. To elucidate RIP biology several questions will have to be addressed, such as how they gain access to their endogenous ribosomes substrates *in vivo* and how they interact with the translation machinery in the cell. The recent identification of RIP from the model system *Nicotiana tabacum* in corroboration with biotechnological approaches, such as mutagenesis and silencing, and genomic approaches, could be an effective way to bridge the gaps in our knowledge. Furthermore, the discovery of novel enzymatic activities could indicate some RIPs' dynamic participation in various cellular mechanisms in response to environmental cues. New insights into the defense mechanisms of RIPs and their additional nonribosomal substrates have also begun to increase our understanding of properties important for protecting organs and regulating cellular functions of host cells. Such varied approaches will likely accelerate our knowledge of the biological function of RIPs and establish their fundamental significance for medical applications in the next few years.

2. Materials

2.1. Plant Material

Candidate plant material is ideally grown under greenhouse or tissue culture conditions. Less fibrous material gives a higher RIP yield. Hairy root culture of some plants is also a good source of RIPs (**10**).

2.2. Composition of Buffers

2.2.1. Plant Extraction Buffer (1 L)

1. 25 mM Phosphate buffer, pH 7.0.
2. 250 mM NaCl.
3. 10 mM Ethylenediaminetetraacetic acid (EDTA).
4. 5 mM Dithiothreitol (DTT).
5. 1 mM Phenylmethylsulfonyl fluoride (PMSF).
6. 1.5% [w/v] Polyvinylpyrrolidone (PVP).

2.2.2. Yeast Ribosome Extraction Buffer

1. 200 mM Tris-HCl, pH 9.0.
2. 200 mM KCl.
3. 25 mM MgCl₂.
4. 25 mM EGTA.
5. 200 mM Sucrose.
6. 25 mM β-mercaptoethanol.

2.3. Ribosome Depurination Assay (All Solutions Must Be Prepared With DEPC-Treated H₂O)

2.3.1. 7 M Urea/6% Polyacrylamide Gel

TBE solution (10X)	0.75 mL
Urea	3.15 g
Polyacrylamide (working solution)	1.5 mL
DEPC-treated H ₂ O	3.0 mL
TEMED	10 μL
APS (10%)	100 μL

2.3.2. Polyacrylamide (Working Solution; 50 mL)

Acrylamide	14.6 g
Bis-acrylamide	0.4 g

2.3.3. TBE (10X; 1 L)

Tris-base	108 g
Boric acid	55 g

EDTA	9.3 g
pH	8.2–8.4

2.3.4. Ethidium Bromide (0.5 mg/mL)

2.3.5. Sample Buffer

Formamide	10 mL
Xilemol cyanol	10 mg
Blomophenol blue	10 mg
0.5 M EDTA	20 μ L
pH	8.0

2.3.6. RIP Buffer (2X; 10 mL)

1 M KCl	334 μ L
0.5 M Tris	400 μ L
1 M MgCl ₂	200 μ L
pH	7.2

Make up to 10 mL with DEPC-treated H₂O.

2.3.7. Extraction Buffer (2X; 100 mL)

1.5 M Tris-HCl, pH 8.8	2.2 mL
NaCl	0.83 g
0.5 M EDTA, pH 8.0	4 mL
10% Sodium dodecyl sulfate (SDS)	20 mL

Make up to 100 mL with DEPC-treated H₂O.

3. Methods

3.1. Protein Extraction From Plant Material

1. Plant materials are ground in liquid nitrogen along with acid-washed sand for maximum cell disruption.
2. The ground material is homogenized in three volumes of extraction buffer (*see Subheading 2.2.1.*) and centrifuged for 30 min at 10,000g.
3. The supernatant is decanted into a clean glass beaker and ammonium sulphate is added to a final concentration of 20% (w/v) with continuous stirring.
4. The mixture is left in the cold room for 1 h and centrifuged for 30 min at 10,000g.
5. The resulting supernatant is precipitated with increasing concentrations of 20–80% (w/v) ammonium sulphate and centrifuged at 14,000g for 30 min.
6. The pellet is suspended in 25 mM HEPES/NaOH, pH 8.0, containing 50 mM NaCl and then dialyzed against 25 mM HEPES/NaOH, pH 8.0, until it is free from the sulphate ions.
7. All extraction procedures are conducted at 4°C, and the ammonium sulphate fraction is stored at –80°C until use.

3.2. Purification of RIP

1. The total protein is fractionated using a cation-exchanger chromatography. Equilibration and loading are carried out using 25 mM NaH₂PO₄ buffer, pH 7.0, at a flow rate of 1 mL/min. The protein was fractionated with NaCl gradient, and further purified using gel filtration chromatography.
2. The gel filtration column is equilibrated with 25 mM HEPES/NaOH, pH 8.0, containing 100 mM NaCl. The basic protein is loaded on the column, and the proteins eluted with an isocratic gradient at a flow rate of 0.5 mL/min.
3. The fractions are assayed for RIP activity, and the active fraction is desalted using 5-kD cutoff ultrafiltration membranes (Millipore, Bedford, MA).

3.3. N-Glycosidase Activity Assay

3.3.1. Isolation of Yeast Ribosomes

Note: All buffers and solutions are prepared using DEPC-treated H₂O.

1. Yeast (*Saccharomyces cerevisiae*) cells are grown in yeast peptone dextrose (YPD) medium at 30°C overnight.
2. Yeast cells are harvested by centrifugation at 4000g for 20 min and washed several times.
3. The pellets are homogenized in extraction buffer (*see Subheading 2.2.2.*) under liquid nitrogen and centrifuged at 11,000g at 4°C for 20 min.
4. The supernatant is laid over a cushion of 10 mL sucrose (1 M sucrose, 20 mM KCl, and 5 mM MgCl₂ in 25 mM Tris-HCl, pH 7.6) in 70-Ti tubes (Beckman Coulter, Fullerton, CA) and centrifuged at 200,000g for 4 h at 4°C (L-70 Ultracentrifuge, Beckman Coulter).
5. The resulting pellet (ribosomes) is resuspended in 25 mM Tris-HCl buffer, pH 7.6, with 25 mM KCl and 5 mM MgCl₂ and stored at -80°C until use.

3.3.2. The Depurination Assay

Note: All buffers and solutions are prepared using DEPC-treated H₂O.

1. Preparation of reaction mixture (final volume: 100 µL).
 - a. 10 µL of ribosome.
 - b. 10 µL 10X RIP buffer.
 - c. Approximately 10–20 µL RIP
 - d. Make up to 100 µL with DEPC H₂O.
2. Incubate the mixture at either 30°C or 37°C for 30 min.
3. RNA is extracted using phenol/chloroform (1:1), and precipitated with ethanol.
4. The pelleted RNA is resuspended with 10 µL of DEPC H₂O.
5. Divide it into two parts (5 µL for aniline treatment, and 5 µL for control); 5 µL of RNA is pipetted into the new Eppendorf tube for control. Store at -80°C until gel electrophoresis.

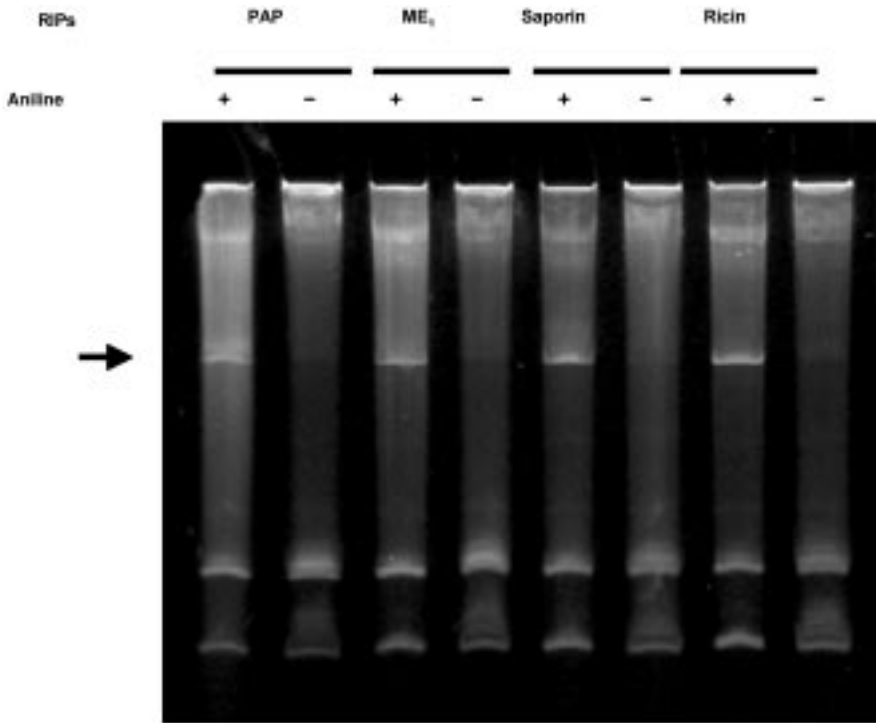


Fig. 1. Enzymatic activity of RIPs in vitro. Ribosomes were isolated from yeast and incubated with PAP (*Phytolacca americana*), ME1 (*Mirabilis expansa*), saporin (*Saponaria officinalis*), and ricin A-chain (*Ricinus communis*) as described in **Sub-heading 3.3.2.**, rRNAs were extracted, treated with aniline, separated on a 7 M 6% Urea polyacrylamide gel, and stained with EB. The presence (+) or absence (-) of aniline is denoted. The arrow shows the presence of the diagnostic 367-nucleotide cleavage product of rRNA.

6. Prepare aniline solution immediately before treatment.
 - a. 400 μ L DEPC H₂O.
 - b. 50 μ L Aniline.
 - c. 60 μ L Glacial acetic acid.
7. Carefully mix the 25 μ L of aniline solution with 5 μ L of RNA using a pipet.
8. Incubate the mixture on ice for 30 min, and RNA is precipitated with ethanol.
9. Add 10 μ L sample buffer (2.3.5) into aniline-treated and control RNAs.
10. Incubate them at 65°C for 10 min.
11. RNAs are separated with 7 M urea/6% polyacrylamide gel.
12. The gel is visualized with ethidium bromide (**Fig. 1**).

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