Increased N-glycosylation of Asn^{88} in serum pancreatic ribonuclease 1 is a novel diagnostic marker for pancreatic cancer

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Alterations of carbohydrate structures in cancer cells are the most promising targets for developing clinical diagnostic reagents. Pancreatic cancer is one of the most difficult cancers to diagnose because it lacks definitive symptoms. Two antibodies were raised against human pancreatic ribonuclease 1 that bind to the enzyme containing unglycosylated Asn^{88}, but not when its Asn^{88} is N-glycosylated. Differential studies using these antibodies in immunoassays and Western blot analyses showed a significant increase in the serum levels of pancreatic ribonuclease 1 containing N-glycosylated Asn^{88} in pancreatic cancer patients compared with normal human subjects. Focusing on the increase in an N-glycosylated Asn residue of serum pancreatic ribonuclease 1, specifically Asn^{88}, affords a new diagnostic marker for pancreatic cancer. This is the first report of a diagnostic cancer marker that takes advantage of the presence or absence of N-glycosylation at a specific Asn residue of a glycoprotein.
Results

An anti-RNase1 antibody with a characteristic reactivity with N-glycosylation site-deficient mutants of RNase1. The N-glycan of RNase1 in serum from patients with PaCa patients is characterized by excess sialylation and core-fucosylation compared with that of healthy donors. To generate a monoclonal antibody (mAb) specific for the N-glycosylation state of RNase1, antibodies were raised in rodents against human RNase1 and subjected to selection using a series of N-glycosylation site-deficient mutants of RNase1. Recombinant mutant proteins that were partially or entirely deficient in N-glycosylation were generated by the substitution of Ala for the third amino acid (Ser or Thr) within the sequon (Table 1, Supplementary Fig. 1). Among the antibodies tested using the ELISA described in Methods, an antibody designated RrhRN0723 failed to react with the mutant proteins designated m110, m010, m100, and m000 (Fig. 1a). In contrast, an antibody designated MrhRN0614 reacted with all mutants as well as with the wild-type RNase1. The shared mutation among the mutants was the substitution of Ser by Ala in the third N-glycosylation site that prevented N-glycosylation of Asn. There are three possible explanations for the lack of binding activity of RrhRN0723 mAb to these mutants. First, RrhRN0723 mAb binds to only N-glycan moieties on Asn. Second, peptide-backbone including Ser residue is essential for binding of RrhRN0723 mAb and the N-glycan on Asn is not involved at all. Third, both Ser residue and N-glycan moieties on Asn are involved in the binding of RrhRN0723 mAb.

Antibody RrhRN0723 recognizes a six amino acid peptide moiety of RNase1 that includes Asn. To determine the role of N-linked glycan on Asn in the reactivity of the RrhRN0723 mAb, a mutant with Thr substituted for Ser (m001T) was generated from m001 and analyzed as described in Methods. Although the m001T mutant has the third N-glycosylation site with Asn as does m001, both the recombinant protein of m001T and with and without glycan linked to Asn were expressed in CHO-K1 cells (Fig. 1b). The substitution of Ser by Thr abolished reactivity with the RrhRN0723 mAb (Fig. 1a), suggesting that this antibody recognizes the Ser residue as part of its binding epitope, and does not recognize only the N-glycan moiety linked to Asn. However, the possibility that the RrhRN0723 mAb simultaneously recognized both the N-glycan and the peptide chain could not be ruled out. Further analyses revealed, however, that substitution of each amino acid residue from positions 85 to 90 impaired the binding of the RrhRN0723 mAb to <50% compared with the wild-type protein, except for the substitution of Thr by the Ser residue (Fig. 1c). Collectively, these findings indicate that the six amino acid peptide moiety of RNase1, Arg-Leu-Thr-Asn-Gly-Ser, represents the minimal epitope essential for the reactivity of the RrhRN0723 mAb (Fig. 1c).

The presence of the N-glycan linked to Asn inhibits the binding of the RrhRN0723 mAb to RNase1. Because the N-glycosylatable Asn is located in the center of the binding epitope, the possibility that the presence of N-glycan linked to Asn may affect the binding of the RrhRN0723 mAb was further investigated. For this purpose, the m001 mutant with only one N-glycan chain linked to Asn was used. The m001 mutant protein expressed in CHO-K1 cells, which was partially N-glycosylated at Asn, was subjected to concanavalin A (ConA) affinity chromatography to separate the N-glycosylated from the unglycosylated form (Fig. 1d left panel). The fractions of ConA column chromatography were analyzed using the ELISA with the RrhRN0723 and MrhRN0614 mAbs (Fig. 1e). N-glycosylated m001 in the ConA-bind fraction (Fig. 1d right panel) was not detected by the RrhRN0723 mAb, in contrast to the MrhRN0614 mAb; although, unglycosylated m001 was detected by both antibodies (Fig. 1e). These results show that the recognition of the six amino acid peptide sequence by the RrhRN0723 mAb was inhibited by the presence of the N-glycan chain linked to Asn.

Analysis of serum RNase1 levels in patients with PaCa using the RrhRN0723 antibody. The levels of RNase1 in sera from healthy donors, patients with PaCa, and patients with other diseases were determined using an ELISA with the MrhRN0614 and RrhRN0723 mAbs (Fig. 2a). The serum level of RNase1 was significantly decreased in patients with PaCa compared with healthy donors and patients with other diseases (Fig. 2b, P = 2.2 × 10^-16). Western blot analyses of serum RNase1 after immunoprecipitation using the MrhRN0614 mAb indicated that the serum RNase1 levels in patients with PaCa were not significantly different compared with those of healthy donors (Fig. 2c). These data indicate that the amount of RNase1 without N-glycosylation of Asn detected by the RrhRN0723 mAb was significantly decreased in the sera of patients with PaCa, although there was no significant difference in the total amount of serum RNase1 of the tested specimens. These results address the hypothesis that the N-glycosylation at Asn of serum RNase1 is increased in patients with PaCa.

Development of an assay to specifically detect unglycosylated Asn in denatured RNase1. To more precisely determine the state of N-glycosylation at Asn of RNase1 in the sera of patients with PaCa, a Western blot analysis was developed (Fig. 3a). Peptide-N-glycosidase F (PNGase F) deamidates N-glycosylated Asn to produce Asp residues by cleaving an N-glycan from a glycoprotein. Serum RNase1 migrates as four distinct bands in an SDS-PAGE gel according to its degree of N-glycosylation. After deglycosylation by PNGase F, the formerly N-glycosylated RNase1 isoforms (top three bands Fig. 3a, left panel) migrate as a single band with an approximate molecular mass of 15 kDa. The anti-RNase1 mAB RN15013, which recognizes the N-terminal peptide sequence,
detects each deglycosylated peptide as a single 15 kDa band. In contrast, an antibody that binds to the peptide moiety by recognizing RNase1 Asn88, but does not bind to the isoform with the Asp88 residue, detects only the RNase1 that originally had an unglycosylated Asn88 in the 15 kDa band (asterisked molecules in Fig. 3a). Therefore, the total RNase1 (differentially glycosylated) and RNase1 with unglycosylated Asn88, named hereafter “Asn88-free RNase1,” can be distinguished on SDS-PAGE gels following PNGase F treatment. The RrhRN0723 mAb did not detect any of the denatured forms of RNase1. Another mAb, RN3F34, was raised that recognized the same peptide epitope of RNase1 as RrhRN0723 (see Methods). The RN3F34 mAb did not detect the upper band of the m001 mutant, which was detected by the RhRN15013 mAb (Fig. 3b), indicating that the RN3F34 mAb did not react with RNase1 that was N-glycosylated on Asn88. Further, RN3F34 did not detect the mutant m000-N88D (Asn88 replaced with Asp, see Table 1 and Supplementary Fig. 1). Therefore, using RN3F34 combined with PNGase F treatment, it was possible to detect Asn88-free RNase1 in serum.

Qualitative analyses of Asn88-free RNase1 in sera of PaCa patients. Qualitative analyses of Asn88-free RNase1 in sera of healthy donors and patients with PaCa were performed using RN3F34 and RhRN15013 after immunoprecipitation with the MrhRN0614 mAb. There was no significant difference in the level of total RNase1 detected by the RhRN15013 mAb between healthy donors and patients with PaCa (Fig. 3c). However, the level of serum RNase1 detected by the RN3F34 mAb in patients with PaCa was significantly less than in healthy donors (Fig. 3c upper panel). These results show that the level of Asn88-free RNase1 was significantly decreased in sera from patients with PaCa, supporting the hypothesis that N-glycosylation at Asn88 of serum RNase1 was significantly increased in the patients with PaCa.

Enzyme immunoassay (EIA) of Asn88-free and total RNase1 for diagnosis of PaCa. The results of EIA analyses for Asn88-free RNase1 using the RhRN0723 mAb did not reliably distinguish patients with PaCa from healthy donors (Fig. 4a center panel, P = 0.594) due to variation in the concentration of total serum RNase1 concentrations.
The present study shows that N-glycosylation at Asn88 of serum RNase1 was significantly increased in patients with PaCa. This finding was established using an immunoassay that was developed to determine the level of total RNase1 compared with that of RNase1 that was not N-glycosylated on Asn88 (Asn88-free). The increase levels of RNase1 with N-glycosylated Asn88 in the sera of patients suspected of having PaCa thus shows promise as a novel diagnostic marker for screening and detection of PaCa. The immunoassay and Western blot-base analysis were developed for detecting this change in N-glycosylation of RNase1 using RrhRN0723 mAb and RN3F34 mAb, respectively. Both antibodies directed against human RNase1 recognize the peptide region surrounding the Asn88 N-glycosylation site containing unglycosylated Asn88, but not when its Asn88 is N-glycosylated. The EIA analysis using RrhRN0723 which allows the high-throughput and quantitative detection of the levels of Asn88-free RNase1 is suitable for clinical purpose. In contrast, the Western blot analysis using RN3F34 which allows the qualitative detection of the changes in N-glycosylation at Asn88 is good for research tool rather than clinical purpose.

The results of the ELISA using this antibody to detect serum RNase1 revealed that the level of RNase1 recognized by the RrhRN0723 mAb was significantly decreased in the sera of patients with PaCa compared with healthy donors or patients with diseases other than PaCa (Fig. 2). There were some patients with PaCa, for example, whose levels of Asn88-free RNase1 were not distinguishable from those of healthy donors, resulting in an apparent decrease in the overall capability of the Asn88-free RNase1 assay (Fig. 4a). Further analyses revealed that the cause was an elevation in the level of total RNase1 in the sera of patients with PaCa (Fig. 4a). Studies conducted during the 1970s and 1980s showed that the serum levels or activity of RNase1 were elevated in patients with PaCa, leading to the conclusion that neither variable served as a specific marker of PaCa. While the results of my present study is similar in some respects to those presented earlier (Fig. 4b, area under curve of the ROC = 0.643 for total RNase1). In contrast, the G3/t ratio described here to measure the N-glycan attached to Asn88 is importantly more accurate and unambiguous in distinguishing patients with PaCa from healthy donors (Fig. 4b).

The functional role of serum RNase1 in association with PaCa is poorly understood, although the elevation of total RNase1 in serum
of PaCa patients has been known from 1970’s14–17. It is also unclear whether the N-glycosylated RNase1 at Asn184 has differential features compared with RNase1 with unglycosylated Asn184, yet. Further analyses will be needed to elucidate the functional role of highly N-glycosylated RNase1 in the development and progression of PaCa.

This study has focused on the presence or absence of N-glycan linked to Asn184 in serum RNase1, in contrast to previous studies describing overall changes in the structure of N-glycan in PaCa cells18–21. Although the structural changes in the N-glycan may be explained by changes in the transcriptional regulation of genes encoding enzymes involved in glycosylation, there is no clear explanation as to why the linked glycosylation of Asn184 in serum RNase1 is increased in PaCa patients. The presence or absence of N-glycan on Asn residues is determined to a large extent by the activity of oligosaccharyltransferase (OST) complexes. OST is a key regulatory enzyme in the linked glycosylation that catalyzes the transfer of a 14 sugar-oligosaccharide moiety from a dolichol-linked precursor to the growing nascent glycoproteins in the lumen of the endoplasmic reticulum (ER)18. Thus, the difference in the degree of N-glycosylation of Asn184, Asn185, and Asn186 in RNase1 may result from the differential activities of OST complexes in a single cell. Up-regulation of the levels of some OST complexes in PaCa cells may increase the level of glycosylation of RNase1 Asn184. Therefore, my future studies will investigate the comparative activities of OST complexes in PaCa and normal cells.

In contrast, preliminary results suggest that this alteration may be due to the structure of RNase1. Among the mutant forms of RNase1 generated to determine the binding epitopes of the RrhRN0723, RrhRN1111, and MrhRN0614, there were mutants that were not recognized by these antibodies. For example, ELISA and Western blot analyses of the His119, Asp121, and Ser123 deletion mutants are shown in Supplementary Fig. 4. In the ELISA analysis, none of these antibodies bound to the mutated RNase1 lacking His119 (Supplementary Fig. 4a). Western blot analyses show that the His119 deletion mutant was heavily N-glycosylated compared with wild-type and the other deletion mutants (Supplementary Fig. 4b).

Because the His119 residue is highly conserved among mammalian pancreatic ribonucleases and is involved in the active site of bovine RNaseA19, the deletion of His119 may possibly induce a conformational change. Thus, the MhrRN0614 mAb, which did not bind denatured RNase1, failed to detect the His119 deletion mutant in the ELISA. Moreover, full N-glycosylation of the His119 deletion mutants, as revealed by Western blot analyses, suggests that a possible conformational change in RNase1 may affect the N-glycosylation of Asn residues. Collectively, these findings are in accord with the hypothesis that a conformational alteration in RNase1 during de novo synthesis in the ER of PaCa cells may enhance the ability of OST complexes to transfer the glycan moiety to Asn184 of RNase1 compared with those of normal cells. The cause of the conformational changes during the de novo synthesis of RNase1 in PaCa cell is unknown, although, ER stress under hypoxic conditions may be one possibility.

Antibodies that form part of the components of IVD reagents used to detect the cancer marker CA15-3 recognize a peptide moiety that includes an O-glycosylation site that is exposed by decreased O-glycosylation of MUC1 that is associated with breast cancer20. Serum transferrin of patients with congenital disorder of glycosylation is under-N-glycosylation and has been used to determine genotypes of deficiency of enzymes involved in glycan biosynthesis21. In these cases, the abnormal absence of the glycan chain is used for detection
of different disease states. In contrast, the present study is the first to identify a cancer marker that distinguishes patients with PaCa by detecting altered levels of N-glycosylation of a specific amino acid residue, Asn88, in RNase1. This “N-glycosylation marker” serves as a cancer marker based on a novel concept of detecting whether a specific N-glycosylatable Asn is N-glycosylated or not.

To date, carbohydrate disease makers including sialyl lewis A (CA19-9), sialyl lewis X and core-fucosylation of alpha-fetoprotein are clinically utilized as IVD reagents. Other structural changes in glycans expressed in cancer cells identified by glycomics research have shown promise as cancer markers2,3. However, there are few examples of exploiting a structural change of an N-glycan revealed by glycomics research that serve as the basis of an immunoassay, which is the most frequently used format for clinical IVD reagents. A possible explanation is the low immunogenicity of glycan chains. In contrast to the conventional glycan markers using anti-carbohydrate antibodies, the advantage of an N-glycosylation marker is that antibody binding requires recognition of the peptide moiety as described herein. Thus, such an antibody will bind to its target with sufficient affinity and specificity for use in an IVD assay compared with antibodies against only the carbohydrate moieties.

Although further clinical research is required to evaluate the suitability for clinical application of the marker for RNase1 described herein, it is likely that it will contribute to the diagnosis and cure of patients with PaCa. In the clinical evaluation researches of this marker started in Tosoh Corporation, it will be particularly important to determine if this type of change in glycosylation is a late or early event in tumor progression as well as its correlation with the clinicopathological factor of patients. The findings reported here should stimulate research to identify similar new markers that may serve to improve the diagnosis and therapy of other diseases.

**Methods**

**Materials.** All synthetic peptides, goat anti human immunoglobulin kappa light chain (hIgk-LC) antibody, recombinant Protein-L and the other chemicals were purchased from Sigma-Aldrich. The MGC cDNA clone of human pancreatic RNase1 was purchased from Thermo Fisher Scientific Inc. The PaCa cell line Capan-1 (ATCC number HTB-79), was obtained from the American Type Culture Collection. Female mice (Balb/c strain) and rats (WKY/NcrlCrlj strain) were purchased from Charles River Laboratories Japan, Inc.

**Serum samples.** Serum samples of patients and healthy donors were obtained from several vendors who collect samples with informed consent under an IRB-approved protocol. Specimens were voluntarily provided by the employees of Tosoh.
Development of anti-human RNase1 antibodies. All experiments using living vertebrates to generate antibodies were performed in accordance with the relevant guideline of Japanese government. Monoclonal antibodies that recognized denatured or intact forms of human pancreatic RNase1 were generated by immunizing mice or rats with synthetic peptides or recombinant proteins, respectively. The hybridoma cell that expresses the antibody RrhRN1111, which binds the N-terminal peptide of denatured RNase1, was generated from B-cells of the retroperitoneal and inguinal lymph nodes of rats immunized with a synthetic peptide corresponding to the N-terminal 14 amino acid residues of RNase1 (KESRKKFFQRQHMDS). This peptide was conjugated to KLH (Thermo Fisher Scientific Inc.) followed by selection using the terminal 14 amino acid residues of RNase1 (KESRKKFFQRQHMDS), which binds to the N-glycosylation site including Asn88 of denatured RNase1, was generated using the same procedures described above, except that the animals were immunized with the synthetic peptide (acyetyl-RLTNGSRYPNC) conjugated to KLH corresponding to Arg85 to Asn94 of RNase1 and the SRLTDGSRYPNC conjugated to BSA for selection of hybridoma cells. The hybridoma cells were selected on the basis of those that produced the desired antibodies that reacted with the intact sequence and not with the peptide in which Asn88 was substituted with Asp. The antibodies which recognize the intact forms of RNase1 were generated using the same procedures as described above, except using spleen B-cells of mice for the MrhRN0614 antibody and B-cells of the retroperitoneal and inguinal lymph nodes of rats that were immunized with recombinant proteins to raise RrhRN0723 and RhRN1111 antibodies. To obtain the recombinant proteins, the insect cell S9 was transfected with the plasmid vector pIZ-KFHhRNase1, which was constructed by inserting the entire higL-βHC-linked to the FLAG and His tags upstream of the RNase1 gene for the MrhRN0614 antibody and B-cells of the retroperitoneal and inguinal lymph nodes of rats that were immunized with recombinant proteins to raise RrhRN0723 and RhRN1111 antibodies. To obtain the recombinant proteins, insect cell S9 was transfected with the plasmid vector pLZ-KFHhRNase1, which was constructed by inserting the entire higL-βHC-linked to the FLAG and 6× His tags upstream of the RNase1 gene. The hybridoma expressing RhRN0614 antibody was purified using an RrhRN0723 antibody-conjugated HiTrap column, followed by freeze-drying under vacuum. For the EIA measurements, the serum was diluted with 90 μl of PBSST followed by mixing with an appropriate amount of MrhRN0614 antibody-beads for 1 h. The beads washed with PBST three times were suspended in 7 μl of PNGase F denaturing buffer and incubated at 37 °C for 1 h. By adding 1 μl of each NF-40 solution, reaction buffer, and PNGase F (New England Biolabs Inc.), then incubating at 37 °C for 1 h, the precipitated RNase1 was released from beads and deglycosylated. The reaction mixtures were divided into two and separated using SDS-PAGE. The PVDF membranes transferred from SDS-PAGE gels were probed with biotin-labeled RhRN15013 and RN3F34 mAbs, respectively.

| Table 2 | Statistical analyses of EIA measurements of serum RNase1 levels of healthy donors and patients with PaCa |
|-----------------|-----------------|-----------------|-----------------|
| specimens       | sample size     | G2/1 ratio      | Asn88-free RNase1 | total RNase1 |
| healthy donor   | 60              | 0.033 (0.014–0.051) | 324.0 (293.5–359.9) | 332.1 (304.8–373.2) |
| PaCa patient    | 91              | 0.159 (0.086–0.290) | 325.7 (219.3–342.0) | 411.3 (297.7–493.0) |
| ROC analysis    | AUC             | 0.795           | 0.481            | 0.643         |
| cutoff          | sensitivity     | 0.089           | 22.0%            | 52.7%         |
| specificity     | specificity     | 75.8%           | 96.7%            | 83.3%         |

*median [interquartile range] The statistical values are from Fig. 4.

ConA-affinity purification of recombinant RNase1. The culture medium of CHO-K1 cells expressing the recombinant KFH-RNase1 m001 mutant was subjected to chromatography using a ConA-conjugated HiTrap column (GE Healthcare UK Ltd.) to separate N-glycosylated from unglycosylated forms using an HPLC system. After washing the column, the N-glycosylated m001 mutant was eluted using a linear gradient of 0-50% of 1M mannosides (0–500 mM). Fraction (1 ml) were subjected to the ELISA described above.

ELISA of pancreatic RNase1 in human serum. The ELISA was carried out as follows: Twenty-five nanograms of the MrhRN0614 mAb in 50 μl PBS was added to a 96-well immunosassay plate and kept at 4 °C overnight. The wells were washed with PBST followed by blocking with 3% BSA in TBS for 2 h. After removing the blocking reagent, diluted human serum was added to each well and kept at room temperature for 1 h. After washing with PBST containing 0.05% Tween-20 (PBST), 25 ng of HRP-conjugated RrhRN0723, RhRN1111, or MrhRN0614 antibodies in PBST containing 1% BSA was added to the wells and kept at room temperature for 1 h. After washing with PBST, the amount of HRP-RrhRN0723 mAb bound to the plate was measured using TMB solution. For the calculation of the amount of RNase1, the RNase1 in the culture medium of Capan-1 cells was purified using an RrhRN0723 antibody-conjugated HiTrap column, followed by the determination of protein amount using the BCA protein assay kit (Thermo Fisher Scientific Inc.). The enzyme immunoassay plate, serially diluted purified RNase1 (0–100 ng/ml) was measured as a standard.

Immunoprecipitation and Western blot analysis of RNase1 from human serum. Immunoprecipitation was performed using the MrhRN0614 mAb adsorbed to Dynabeads protein G beads (Life Technologies). Filter papers of human serum was diluted with 90 μl of PBST followed by mixing with an appropriate amount of MrhRN0614 antibody-beads for 1 h. The beads washed with PBST three times were suspended in 7 μl of PNGase F denaturing buffer and incubated at 37 °C for 1 h. By adding 1 μl of each NF-40 solution, reaction buffer, and PNGase F (New England Biolabs Inc.), then incubating at 37 °C for 1 h, the precipitated RNase1 was released from beads and deglycosylated. The reaction mixtures were divided into two and separated using SDS-PAGE. The PVDF membranes transferred from SDS-PAGE gels were probed with biotin-labeled RhRN15013 and RN3F34 mAbs, respectively.

ELA detection of serum RNase1. To measure the levels of Asn88-free and total RNase1 in serum, two ELA reagents for use with an Automated Immunoassay Analyzer (AIA, Tosoh Corporation, Japan) were developed. The AIA and its test cup are shown in Supplementary Fig. 3. Briefly, for total RNase1 detection, a new anti-RNase1 antibody, RhRN1111, which bound serum RNase1 independently of the state of N-glycosylation, was used. To prepare ELA reagents for Asn88-free RNase1 or for total RNase1 measurements, the test cups for the AIA reagent containing the MrhRN0614 mAb (270 ng per test cup) adsorbed to the magnetic carrier beads were added the alkaline phosphatase-conjugated RrhRN0723 mAb (50 ng per test cup) or to the RhRN1111 mAb (50 ng per test cup) in 100 μl of PBST containing 1% BSA followed by freeze-drying under vacuum. For the ELA measurements, the serum specimens were diluted with 20 volumes of TBS containing 1% BSA. Using two AIA reagents with the AIA-2000 analyzer, the amount of Asn88-free and total RNase1 in the sera of healthy donors and patients with PaCa were determined. The concentrations of each analyte were determined according to the calibration curves of serially-diluted RNase1 (0.0–100 ng/ml for Asn88-free RNase1, 0.0–106.5 ng/ml for total RNase1) expressed and purified from CHO-K1 cells, and the concentration was determined using authentic Asn88-free RNase1. The calibration curves for both analyses are shown in Supplementary Fig. 5. Statistical analyses were carried out using R statistic software with the pROC suite for ROC analysis.
Materials availability. The antibodies, MrhRN0614, RrhRN0723, RrhRN1111 and RN3F34 in this article are available for research-only purposes from Tosoh Corporation with the restrictive Material Transfer Agreement. The AIA reagents for detecting Asn™-free and total RNase1 will be available for research-only purposes from Tosoh Corporation.

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