
http://dx.doi.org/10.1038/nprot.2014.047

Nature Publishing Group

Author's final manuscript

Thu Jul 05 22:26:54 EDT 2018

http://hdl.handle.net/1721.1/99341

Article is made available in accordance with the publisher's policy and may be subject to US copyright law. Please refer to the publisher's site for terms of use.

Please share how this access benefits you. Your story matters.
Quantitative analysis of tRNA modifications by HPLC-coupled mass spectrometry

Dan Su1,§, Clement T.Y. Chan1,¶, Chen Gu1, Kok Seong Lim1,†, Yok Hian Chionh2, Megan E. McBee2, Brandon S. Russell1, I. Ramesh Babu1, Thomas J. Begley3, and Peter C. Dedon1,2,4,*

1Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA
2Singapore-MIT Alliance for Research and Technology, CREATE, Singapore
3College of Nanoscale Science and Engineering, University at Albany, State University of New York, Albany, NY
4Center for Environmental Health Science, Massachusetts Institute of Technology, Cambridge, MA

Abstract

Post-transcriptional modification of RNA is an important determinant of RNA quality control, translational efficiency, RNA-protein interactions, and stress response. This is illustrated by the observation of toxicant-specific changes in the spectrum of tRNA modifications in a stress response mechanism involving selective translation of codon-biased mRNA for critical proteins.

To facilitate systems-level studies of RNA modifications, we developed a liquid chromatography-coupled mass spectrometry (LC-MS) technique for the quantitative analysis of modified ribonucleosides in tRNA or other RNA species. The protocol includes tRNA purification by HPLC, enzymatic hydrolysis, reversed-phase HPLC resolution of the ribonucleosides, and identification and quantification of individual ribonucleosides by LC-MS using dynamic multiple reaction monitoring. This approach enables quantification of modified ribonucleosides in several micrograms of tRNA, or other RNA, in a 15-minute LC-MS run. By comparison, traditional

*Correspondence should be addressed to Peter Dedon, Department of Biological Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, 56-787B, Cambridge, MA 02139; pcedon@mit.edu.

§Present address: Life Technologies, 246 Goose Lane, Suite 100, Guilford, CT 06437
¶Present address: Department of Biomedical Engineering, Boston University, Boston, MA
†Present address: Technology Group, LLC, 20 Park Plaza, Suite 1200, Boston, MA 02116


AUTHOR CONTRIBUTIONS STATEMENTS

All authors contributed to experimental design, data analysis and interpretation, and preparation of the manuscript. D.S., C.T.Y.C., C.G., K.S.L., Y.H.C., B.S.R., M.E.M., I.R.B. contributed to the development of the protocol and performed experiments that contributed to this manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.
methods for detecting modified ribonucleosides are labor and time intensive, require larger RNA quantities, are modification-specific, or require radioactive labeling.

Search terms
ribonucleoside; secondary modification; tRNA; chromatography; mass spectrometry; RNA purification

INTRODUCTION

Most forms of RNA in prokaryotic and eukaryotic cells contain modified ribonucleosides in addition to the canonical adenosine, guanosine, cytidine and uridine, with more than 100 different chemical structures across all organisms and 2–3 dozen in any one organism (structures of several modified ribonucleosides in tRNA from *Saccharomyces cerevisiae* are shown, for example, in Figure 1).1-6 tRNA is the most heavily modified RNA species, with the presence of specific ribonucleoside structures affecting the rate and fidelity of translation,3-6 tRNA stability,7,8 cellular stress responses9-11 and cell growth.12 However, emerging evidence points to a systems-level function of the complete set of modified ribonucleosides. For example, several recent studies have revealed a role for tRNA modifications in controlling cellular stress response and phenotype by directing the selective, codon-biased translation of specific mRNAs,10,13,14 with unique changes in the levels of modified ribonucleosides for different stimuli and stresses.10,14,15

The discovery of this emergent property of tRNA modifications has been enabled in part by application of liquid chromatography-coupled mass spectrometry (LC-MS) to quantify the complete set of tRNA modifications and how its composition varies in response to different stresses. Traditional approaches to quantifying individual modified ribonucleosides, such as two-dimensional thin-layer chromatography16,17, are semi-quantitative at best and not suitable for studying all tRNA modifications at once, in addition to being labor and time intensive and employing radioactive labeling. Mass spectrometry-based methods have recently emerged as powerful tools for identifying and quantifying RNA modifications.18-27 For example, we recently developed a rigorously quantitative platform for LC-MS analysis of the system of modified ribonucleosides in the population of tRNA molecules in a cell or in individual, purified tRNA species (Figure 2), with the method applied to studies in bacteria, yeast and human cells.14,15,28-30 Application of this approach to the identification and quantification of 25 tRNA ribonucleoside modifications in *Saccharomyces cerevisiae* led to the discovery of a direct link between cell stress and translation of stress-response proteins.14,15 As illustrated in Figure 2, the basic approach involves 5 stages: (1) tRNA purification and quantification; (2) hydrolysis of tRNA to individual ribonucleosides; (3) reversed-phase HPLC resolution of ribonucleosides; (4) identification and quantification of ribonucleosides by tandem quadrupole mass spectrometry (QQQ); and (5) multivariate statistical analysis of patterns of change in the set of modified ribonucleosides caused by the stress or stimulus. Using this approach as an exploratory tool, we discovered that yeast respond to different chemical stresses with agent-specific changes in the relative quantities of the two-dozen modified ribonucleosides in tRNA, or ‘reprogramming’, which leads to
selective translation of codon-biased mRNAs representing proteins that are critical for the stress response to the specific chemical stressor. Here we present a protocol for the LC-MS platform for quantitative analysis of modified ribonucleosides in total tRNA isolated from a cell. The data are used to assess population-level changes in the relative quantities of the ribonucleosides regardless of whether the changes occur by alterations in tRNA copy number, the activity of tRNA modifying enzymes or both. The patterns revealed by this approach provide clues for subsequent analysis of individual tRNA species and tRNA copy numbers.

One key advantage of the LC-MS protocol described herein with respect to alternative methods is the highly quantitative nature of the approach. This characteristic is critical, as it enables researchers to identify not only the presence or absence of certain modifications in mutants lacking modification pathways, but, more importantly, to quantify changes in modification levels that result from altered regulatory pathways in cells, a task that is very difficult to perform using traditional methods. For example, using the present protocol, we found that oxidative stress caused an increase in the level of 5-methylcytidine (m5C) and a reduction in the level of 5-methoxycarbonylmethyl-2-thiouridine (mcm5s2U) in total tRNA one hour after exposing yeast to hydrogen peroxide (Figure 3A, structures in Figure 1). Having identified ribonucleosides for which stress-induced changes are substantial, we then used established approaches, such as RNase-based mass spectrometric mapping of modified ribonucleosides in specific tRNAs, to quantify changes in the level of m5C at the wobble position of tRNALeuCAA that led to selective translation of genes enriched in TTG codons.

Although the in vivo kinetics of tRNA modification have not been measured, the observation of changes in the levels of tRNA modifications within an hour of cell exposure points to a regulatory system involving increased enzyme activity and/or increased tRNA copy numbers, or degradation of tRNA species and possible enzymatic removal of modifications. For instance, oxidative demethylation, which is known to occur in DNA with 5-methyl-2-deoxycytidine, may also take place in tRNA, as suggested by the presence of demethylation motifs in tRNA modifying enzymes such as ABH8.

An important feature of this quantitative approach is that it can be applied to any type of coding and non-coding RNA, when coupled with an appropriate RNA purification technique. The implementation of such a technique is especially important in light of the presence of modified ribonucleosides in virtually every form of coding and non-coding RNA in all living organisms. A critical requirement of the present approach is the isolation of specific RNA species free from contamination of other RNA species or RNA fragments, so as to avoid the wrongful identification of modifications that do not exist in the species of interest. A variety of chromatographic and non-chromatographic methods exist for purifying non-coding RNA species, including our recently developed multidimensional HPLC method, with polyA affinity purification methods for mRNA species. Here, we used the HPLC approach for tRNA purification.

**Experimental design**

Several crucial features of the experimental design are beyond the scope of the Procedure detailed below, yet they play a critical role in the outcome of the experiments. The first is
the quality and accurate quantification of the purified RNA subjected to LC-MS. Most commercial kits do not provide the purity of tRNA or other RNA molecules necessary for truly precise and accurate quantification of modified ribonucleosides in the RNA species of interest. For example, small RNA isolation kits yield RNA species up to approximately 200 nt, with tRNA representing 80–95% of the purified tRNA\textsuperscript{15} and with potential contamination with 5S and 5.8S rRNA, rRNA degradation products, and microRNA species. Further purification of tRNA by chromatographic methods\textsuperscript{52} or acrylamide gel electrophoresis\textsuperscript{14} reduces the risk of contamination problems. We have successfully used both approaches, with HPLC purification employed in the present procedure.\textsuperscript{10,14,15,30} Furthermore, different RNA isolation methods have been shown to bias the isolation toward large or small RNA species and to different sequence enrichments in the RNA\textsuperscript{53–55}. The isolation and purification method could also lead to artifacts caused by oxidation or enzymatic deamination, such as is known to occur in DNA, which can be reduced by incorporation of antioxidants and deaminase inhibitors\textsuperscript{56}. For example, care must be taken to avoid enzymatic deamination of adenosine to inosine by deaminases that contaminate some preparations of alkaline phosphatase\textsuperscript{56,57}, and to avoid loss of redox-sensitive modifications, such as the loss of 5-hydroxycytidine observed in prokaryote rRNA\textsuperscript{58}. Finally, precise and accurate quantification requires both biological and technical replicates of the analyses. In general, experiments should be performed with technical duplicate analyses of each of three biological replicates for each condition, along with untreated cells.

Another important prerequisite to the analysis of stress-induced reprogramming of tRNA modifications is the identification of the complete set of modified ribonucleosides in an organism. The current databases are focused on individual modifications discovered over decades of study, mainly for \textit{Escherichia coli}, yeast and human cells.\textsuperscript{2,22} This means that most modified ribonucleosides are known for these organisms, but few others. Even with ~70% conservation of the various structures across all organisms, there is still the need to identify the HPLC elution behavior of the modified ribonucleosides from well-studied organisms, which requires the use of commercial standards (see Materials) or the identification of ribonucleosides by high mass-accuracy MS, collision-induced dissociation MS and NMR spectroscopy.

Finally there is the issue of instrument setup. We have used Agilent UPLC-coupled mass spectrometry systems, and the methods we describe here can be readily translated to LC-MS systems from other manufacturers. The parameters in need of attention are settings of the source (ionization energy, temperature, gas flow, \textit{etc.}), the analyzer (collision energy, gas flow, \textit{etc.}) and the detector (mode of detection, exact monitored mass transitions, \textit{etc.}). We used the dynamic multiple reaction monitoring (DMRM) program available on the Agilent instruments to achieve higher sensitivity, and similar programs are available for other modern QQQ instruments. For example, AB Sciex QQQ instruments have a feature called “scheduled MRM,” whereas Thermo Fisher QQQ instruments have “timed-SRM.” These software programs all work in a similar fashion to optimize the sensitivity of detecting individual ribonucleosides with known structure and mass. For older instruments that lack such programs, multiple reaction monitoring (MRM) can be set up to achieve the same goal, though with reduced sensitivity.
Perhaps the most critical stage in this LC-MS platform for quantitative comparison of changes in modified ribonucleosides across samples is the quantification of RNA in each sample. Standard spectroscopic methods (i.e., A$_{260}$) are entirely inadequate for this purpose, as they are heavily affected by contamination with even small amounts of protein, DNA, buffers and other chemicals. However, even highly precise and accurate RNA quantification methods, such as fluorescent dye binding, will not provide accurate information about the RNA content of the sample if the method is applied too early in the sample processing. Notably, loss of RNA in subsequent HPLC purification and enzymatic digestion steps will cause artifacts in RNA quantification and wide variations in data from replicate samples. The optimal solution to these issues is to quantify the canonical ribonucleosides (i.e., cytidine, uridine, adenosine, and guanosine) in the fully processed sample at the time of LC-MS analysis, which can be achieved using an in-line UV absorbance detector, with an external calibration curve. Alternatively, one can quantify the canonical ribonucleosides by MS in a second run on the instrument with a dilution of a small portion of the original sample in cases where the in-line UV absorbance detector is not available. Either approach will provide the most accurate measurement of input RNA concentration by which to normalize the MS signals for the individual modified ribonucleosides in comparisons across different samples, which obviates problems with protein and DNA contamination. Here, we used the in-line UV diode array detector module coupled to an Agilent 1290 UPLC to quantify the canonical ribonucleosides. To avoid problems caused by day-to-day variation in instrument performance, the precision of relative quantification of modifications across different analysis sessions may be enhanced by spiking into the fully processed analyte an internal standard, such as $^{15}$N$_5$-2-deoxyadenosine ($^{15}$N-dA) to a final concentration of 40 nM immediately prior to MS analysis. If all samples are analyzed on the same day, this internal standard approach is not necessary.

Absolute quantification of individual modified ribonucleosides can be achieved by preparing external calibration curves using a synthetic standard of the ribonucleoside. However, this is beyond the scope of this protocol.

**Limitations**

As with all methods, there are limitations to this LC-MS platform and many of them have already been discussed. Other limitations include the level of sensitivity of the LC-MS protocol, which, even with optimized RNA isolation and analytical parameters, can result in the inability to detect and quantify rare modified ribonucleosides. For example, of the 25 tRNA modifications known to be present in yeast, we were not able to detect 2′-O-ribosyladenosine phosphate (Ar(p)) in positive ion mode, possibly due to the strong negative charge of the phosphate, and we made only tentative identification of 5-Carbamoylmethyl-2′-O-methyluridine (ncm$^5$Um) by collision induced dissociation (CID) due to weak signal intensities.

In spite of the few limitations of the method, the following LC-MS platform provides highly precise quantification of changes in the spectrum of modified ribonucleosides in tRNA from any organism in studying translational control of cellular response and phenotype.
MATERIALS

REAGENTS

**Ribonucleosides to be used as HPLC standards**

- 2’-O-Methylguanosine (Berry & Associates, cat. no. PR 3760)
- N4-Acetylcytidine (Advanced Molecular Technologies Pty Ltd, cat. no. NS005-A05)
- 2’-O-Methyladenosine (Sigma, cat. no. M9886)
- 2’-O-Methyluridine (Berry & Associates, cat. no. PY 7690)
- 2’-O-Methylcytidine (MP Biomedicals, cat. no. 153856)
- N2-Methylguanosine (Sigma, cat. no. M4004)
- N2,N2-Dimethylguanosine (Berry & Associates, cat. no. PR 3702)
- 1-Methyladenosine (Berry & Associates, cat. no. PR 3032)
- Inosine (Berry & Associates, cat. no. PR 3725)
- 5-Methyluridine (Sigma, cat. no. 535893)
- N6-Isopentenyladenosine (AAA Chemistry, cat. no. AR-1J8622)
- 7-Methylguanosine (Sigma, cat. no. M0627)
- N6-Methyladenosine (Berry & Associates, cat. no. PR 3732)
- 5-Methylcytidine (Berry & Associates, cat. no. PY 7637)
- Pseudouridine (Berry & Associates, cat. no. PYA 11080)
- 3-Methylcytidine (Sigma Chemical Co., cat. no. M4949)
- N6-Threonylcarbamoyladenosine (Biolog, Bremen, Germany, cat. no. C 022)
- [15N]5-2-deoxyadenosine (Cambridge Isotope Laboratories, cat. no. NLM-3895)

**Other reagents**

- Acetonitrile (VWR, cat. no. BJ015)
- Alkaline phosphatase (17 U µl−1; Sigma Chemical Co., cat. no. P5521)
- Ammonium acetate (Sigma, cat. no. A1542)
- Ethanol (Pharmco, cat. no. 111ACS200)
- Formic acid (Fluka, cat. No. 94318))
- Glacial acetic acid (Aldrich, cat. no. 317276)
- HPLC-grade water (VWR, cat. no. BJ365)
- Phosphodiesterase I (US Biological, cat. no. 20240Y)
- Benzonase (Sigma, cat. No. E8263)
- TRIReagent (Sigma, cat. No. T9424)
- Chloroform (>99.5%)
- Buffer Y1 (1 M Sorbitol, 100 mM EDTA pH 8.0)
- B-Per (Thermo Fisher Scientific, cat. no. 78266)
- Lyticase (Sigma, cat. no. L2524)
- Glycogen (Roche Applied Science, cat. no. 10901393001)
- Zymolase (Zymo Research, cat. no. E1004)
- Trizma (ACS grade)
- Magnesium chloride (ACS grade)
- Sodium acetate (ACS grade)
- 10× PBS (Sigma, cat. no. P5493-1L)
- β-mercaptoethanol (Sigma, cat. no. M6250)
- RNase-free H₂O (Qiagen, cat. no. 129114)
- 100% Isopropanol
- E. coli, S. cerevisiae and mammalian cells were obtained from American Type Culture Collection (ATCC) and cultured according to protocols provided by ATCC.

EQUIPMENT
- 15-ml Falcon tubes (VWR, cat. no. 21008-918)
- 50-ml Falcon tubes (VWR, cat. no. 21008-951)
- A triple quadrupole mass spectrometer system (QQQ Yes), such as the Agilent 6430 (Agilent Technologies)
- A UPLC system, such as the Agilent 1290 (Agilent Technologies)
- Agilent Qualitative Analysis software (Agilent Technologies)
- Agilent 1100 Series HPLC with fraction collector
- Agilent SEC-3 300Å HPLC column (Agilent Technologies, cat. no. 5190-2511)
- Autoclave
- Autosampler (Agilent Technologies)
- Avanti J-25 centrifuge (Beckman)
- Bioanalyzer (Agilent Technologies)
- Blue screw caps (Agilent Technologies, cat. no. 5182-0717)
- Digital heat block (VWR)
• Diode array detector, G1315B (Agilent Technologies)
• DU-640 UV-visible spectrophotometer (Beckman)
• HPLC column heater (Agilent Technologies)
• Hypersil GOLD aQ 3-µm 150 × 2.1 mm HPLC column (Thermo, cat. no. 25003-152130)
• Microcon YM-10 centrifugal filtration cartridge (Millipore, cat. no. 42407)
• 10,000-MW cut-off spin filter (Amicon, cat. no. UFC501024)
• Razors
• Scissors
• 2-ml Screw top vial (Agilent Technologies, cat. no. 5182-0715)
• Vial insert tube, 100-µl glass with polymer feet (Agilent Technologies, cat. no. 5181-1270)

REAGENT SETUP

UPLC-MS mobile phases To prepare fresh mobile phase A, mix 100% HPLC-grade water with the relevant amount of formic acid to achieve a final formic acid concentration of 0.1% (vol/vol); to prepare mobile phase B mix 100% acetonitrile with the relevant amount of formic acid to achieve a final formic acid concentration of 0.1% (vol/vol).

Buffer Y1 (yeast lysis buffer) To prepare 1 liter of buffer Y1, add 182.17 g of sorbitol to ~500 ml of RNA-free water, then add 200 ml of 0.5 M EDTA (pH 8.0) into the solution, mix until the solution is clear, and add RNA-free water to 1 liter. Store at 4 °C. Add β-mercaptoethanol to a final concentration of 14 mM immediately before use.

3M sodium acetate (pH 5.2) Dissolve 408.24 g of sodium acetate•3H$_2$O in 800 ml of water. Adjust the pH to 5.2 with glacial acetic acid and then adjust the volume to 1 liter with water.

! CAUTION All solutions should be prepared in a fume hood.

! CAUTION Buffer Y1 should be prepared at least monthly.

CRITICAL Because of possible bacterial growth in solutions, prepare fresh mobile phase A before each run. Mobile Phase B can be stored at ambient temperature (22 °C in our lab) indefinitely due to the high content of organic solvent.

EQUIPMENT SETUP

HPLC setup—For HPLC set up (operating parameters, solvent parameters, etc.), follow the manufacturers’ instructions for instrument operation, with particular attention paid to the parameters specific to the experiments performed in this protocol. HPLC column equilibration for the size-exclusion step for tRNA purification and the reversed-phase resolution of ribonucleosides is described in specific sections in this protocol and parameters for experiments described in this protocol shown in the table in Step 51.
Mass spectrometer setup—Follow the manufacturer’s instructions for instrument setup and operation; basic mass spectrometer settings for the studies presented here are noted in the table below. Instructions for optimizing CID (collision-induced dissociation) parameters (e.g., collision energy and fragmentor voltage) of standard nucleoside measurements using the MassHunter Optimizer, which is included in the software package installed with all Agilent QQQ systems, are presented in Step 47. Instructions for determining the appropriate HPLC retention time values for DMRM analysis of ribonucleosides (Table 1) are described in Step 51.

<table>
<thead>
<tr>
<th>Mass spectrometer parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas temperature (°C)</td>
<td>350</td>
</tr>
<tr>
<td>Gas flow (l/min)</td>
<td>10</td>
</tr>
<tr>
<td>Nebulizer gas (psi)</td>
<td>20</td>
</tr>
<tr>
<td>Capillary voltage (V)</td>
<td>3500</td>
</tr>
<tr>
<td>Delta EMV (Electron Multiplier Voltage) (V)</td>
<td>650</td>
</tr>
</tbody>
</table>

**PROCEDURE**

**Isolation of total RNA**

1| Prepare cultures of *E. coli*, *S. cerevisiae* or mammalian cells to yield 50–100 µg of total RNA. This amounts to roughly 100 ml of mid-log phase *E. coli* (~10^9 cells) or *S. cerevisiae* (~10^8 cells), and 10^6–10^7 adherent or suspension cultures of mammalian cells. These values are rough guidelines for experimental design and should produce adequate RNA for tRNA purification and quantification of modified ribonucleosides, but yields will vary.

2| Pellet the cells by centrifugation (1000 ×g for 5 min for mammalian cells, 3000 ×g for 10 min for *E. coli* and *S. cerevisiae*) at 4 °C. Remove culture medium by aspiration, wash the cells with 1× PBS or water as needed to remove any treatment reagents, and snap freeze the pellet in liquid nitrogen. Store at −80 °C.

▲CRITICAL STEP It is important to control the exposure conditions and exposure times carefully by snap freezing cell pellets in liquid nitrogen in studies or immediately denaturing the samples with RNA extraction reagents in studies of exposure-induced changes in tRNA modification levels, steps 3 to 17 in this protocol. The activities of RNA-modifying enzymes will not cease until the proteins are frozen or denatured, and the levels of modified ribonucleosides will definitely change during prolonged incubations and processing steps unless care is taken to stop the relevant reactions.

■ PAUSE POINT The cell pellets can be stored at −80 °C for several weeks.

3| Lyse cells according to Option A, Option B, or Option C, depending on whether your cells pellets from step 2 are from *E. coli*, *S. cerevisiae*, or human cells, respectively.

**Option A. Lysis of *E. coli* cell pellet**
i. Resuspend the pellet in 5 ml of B-Per, vortex and allow to stand at ambient temperature (20–22 °C), for 10 min. The solution should be clear.

**Option B. Lysis of S. cerevisiae cell pellet**

i. Resuspend the cell pellet in 5 ml of freshly prepared Buffer Y1 (in the presence of 0.1% or 14mM β-mercaptoethanol immediately before use) containing lyticase or zymolase (50 U per ml of cells). Incubate for 10–30 min at 30 °C with gentle shaking to generate spheroplasts. Spheroplasts must be handled gently.

**Option C. Lysis of mammalian cell pellet.**

i. Resuspend the cell pellet in 5 ml of RNase-free H2O and vortex quickly to break up the pellet.

4| Add 25 ml of TRIReagent to the cell lysate, vortex and allow to stand at ambient temperature for 5 min.
5| Add 5 ml of chloroform to the mix and allow the mixture to stand at ambient temperature for 3 min.
6| Centrifuge the sample at 12,000 ×g for 15 min at 4 °C.
7| Transfer the aqueous phase to a new tube.
8| To the aqueous phase, add 0.5 ml of 100% isopropanol per ml of TRIReagent used for homogenization.
9| Incubate the tube from step 8 at ambient temperature for 10 min.
10| Centrifuge at 12,000 ×g for 10 min at 4 °C.
11| Remove the supernatant to leave an RNA pellet.
12| Wash the pellet with 1 ml of 75% (vol/vol) ethanol in water per ml of TRIReagent used in the initial homogenization. Vortex the sample briefly, then centrifuge the tube at 7500 ×g for 5 min at 4 °C. Discard the supernatant washing solution.
13| Repeat step 12 once more.
14| Air-dry the RNA pellet for 5–10 min.
15| Resuspend the RNA pellet in RNase-free water and measure the RNA concentration by A260 or other means. Generally, ~100 µg of total RNA can be extracted from 100 ml of yeast culture. Troubleshooting (Table 2).
16| Check the quality of the isolated total RNA by running a fraction on a Bioanalyzer, or by gel electrophoresis. See the example of an Agilent Bioanalyzer output for total RNA from human cells in Figure 4. Visual analysis of the gel or image will reveal gross degradation, with loss of the individual peaks for tRNA and rRNAs. More subtle degradation can be detected using the RNA Integrity Number (RIN), which is related to the 28S/18S ratio, calculated

---

*Nat Protoc. Author manuscript; available in PMC 2015 February 02.*
by the Agilent Bioanalyzer. This value estimates the quality of the RNA and can be referenced against the RNA Integrity Database (RINdb) available at http://www.chem.agilent.com/rin/_rinsearch.aspx. Low quality RNA could be due either to sample handling, which necessitates repeating the experiment, or the cellular stress response, which indicates the need to use lower levels of stimulant or toxicant exposure. Troubleshooting (Table 2).

17| Aliquot 10–50 µl and store total RNA at −80 °C until further use.

■ PAUSE POINT The purified RNA can be stored at −80 °C for at least 3 months.

Isolation of total tRNA from total RNA by HPLC

18| Completely thaw an aliquot of total RNA on ice and, immediately proceed to the next step.

19| Denature the RNA sample by incubating it on a heating block at 70 °C for 2 min, then place it on ice for at least 5 min.

CRITICAL STEP Failure to heat-denature RNA before loading the solution to the HPLC device would result in very wide peaks and little separation.

20| Measure RNA concentration by absorbance at 260 nm and 320 nm. The absorbance at 260 nm should be corrected by subtracting absorbance at 320 nm, which is used as the background of the measured spectrum. Measured with a 1-cm path length, 1 absorbance unit at 260 nm (A_{260} unit) is roughly equivalent to 40 µg/ml of RNA.

21| If the RNA concentration is less than 5 mg/ml, concentrate total RNA on a Microcon YM-10 centrifugal filtration cartridge following manufacturer’s instructions or a similar system.

22| Transfer 50 µg of total RNA (<10 µl volume) into a 100-µl vial insert tube, place the insert in a 2-ml screw top vial and cap the vial.

CRITICAL STEP After transferring the RNA to the insert tube, check for bubbles at the bottom of the insert tube and use a pipette to dislodge or remove any bubbles, the presence of which would interfere with the HPLC-based purification of the tRNA.

23| Equilibrate an Agilent SEC-3 300Å HPLC column (300 mm length × 7.8 mm inner diameter) on Agilent 1100 Series or a similar HPLC system with a temperature-controlled column compartment at 65 °C with 100 mM ammonium acetate aqueous phase at a flow rate of 0.5 ml/min for at least 20 min.

24| To determine the retention time of the tRNA, inject 1 µl of total RNA into the HPLC according to the directions in Step 22.

25| Record the retention time and peak width of the tRNA signal.

26| Inject the remaining total RNA solution into the HPLC system and collect the tRNA peak using the retention times noted in Step 25. Collection of the tRNA
can be performed manually or by programming the HPLC with a fraction collector.

**CRITICAL STEP** Be careful not to overload the HPLC column. The Agilent SEC-3 300Å HPLC column has a maximal capacity of ~100 µg of RNA.

27] Mix the volume of collected tRNA with 0.1 volumes of 3 M sodium acetate (pH 5.2) and then add 2.5 volumes of ice-cold 100% ethanol and 20 µg of glycogen. Invert the tube a few times to fully mix the contents.

28] Place the tube at −80 °C for at least 20 min.

29] Centrifuge the tube at 14,000 ×g for 30 min at 4 °C. A white pellet should appear at the bottom of the tube.

30] Remove the supernatant carefully and add an equal volume of 70% (vol/vol) ethanol in water to the tube. Mix thoroughly.

31] Centrifuge the tube for at 14,000 ×g for 5 min at 4 °C and again discard the supernatant.

32] Repeat steps 29 and 30.

33] Air-dry the precipitated tRNA for 5–10 min at ambient temperature.

34] Dissolve the dried tRNA in 20 µl of RNase-free water.

35] Measure the RNA concentration as in step 20.

36] Remove 1 µl of total tRNA and dilute it with RNase-free water to a concentration of 50–500 ng/µl.

37] Analyze the diluted sample with an Agilent Bioanalyzer small RNA chip to determine the integrity and purity of purified total tRNA. The sample should be completely free of microRNA, 5S rRNA and other contaminants. An example of Agilent Bioanalyzer analysis of total RNA and purified total tRNA from human cells is shown in Figure 3.

■ **PAUSE POINT** The purified tRNA can be stored at −80 °C for at least 3 months.

**Hydrolysis of tRNA to nucleosides and dephosphorylation of nucleosides**

38] Place 10 µg of total tRNA in a microcentrifuge tube on ice.

39] Using the volumes specified in the following table, prepare a mixture containing all of the enzymes and cofactors needed for hydrolysis of the RNA into nucleosides.

<table>
<thead>
<tr>
<th>Stock solution of reagent</th>
<th>Volume added (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 mg/ml BSA</td>
<td>25</td>
</tr>
<tr>
<td>5 U µl⁻¹ of benzonase</td>
<td>10</td>
</tr>
</tbody>
</table>
Add 10 µl of the mixture prepared in step 39 to each sample of tRNA and bring the final volume to 50 µl with RNase-free water.

Incubate the samples at 37 °C for 3 h.

Enzyme removal

Rinse a 10,000-MW cut-off spin filter (Amicon) by adding to it 300 µl of deionized water and centrifuging for 5 min at 16,000 ×g at 4 °C.

Place each rinsed spin filter onto a fresh collection tube.

Transfer the hydrolyzed RNA sample to the rinsed spin filter and centrifuge for 10 min at 16,000 ×g at 4 °C.

Transfer the filtrate from Step 44 to a 100 µl vial insert tube, place in a 2-ml screw top vial and cap the vial. These samples will be analyzed in Step 55.

CRITICAL STEP After the RNA has been transferred to the RNA to the insert tube, check for bubbles at the bottom of the insert tube and use pipette to dislodge or remove any bubbles.

■ PAUSE POINT The samples in vials can be stored at −20 °C for at least 2 weeks and for longer at −80 °C.

Optimize the mass spectrometer parameters for targeted ribonucleosides

Using an appropriate set of the commercial ribonucleoside standards listed in the Reagents section (most are present in all organisms), use a stock solution of each ribonucleoside to prepare a 50-µl solution at a final concentration of ~0.2 ng/µl in a 100-µl vial insert tube inserted into a 2-ml screw top vial. Cap the vial. Proceed to make multiple injections of the same sample, using different injection volumes from 1 to 10 µl. The ability to detect a roughly linear (non-linear is acceptable) increase in peak area for each ribonucleoside provides an estimate of the dynamic range for detecting the ribonucleosides.

Connect a column-free UPLC system directly to a QQQ mass spectrometer with 0.1% (vol/vol) formic acid as the solvent and set up the MassHunter Optimizer program using the parameters listed below.

<table>
<thead>
<tr>
<th>Optimizer parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC</td>
<td></td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.4 ml min⁻¹</td>
</tr>
</tbody>
</table>
Initiate the MassHunter Optimizer program. The UPLC system autosampler will inject a volume of the sample into the mass spectrometer and determine optimal ionization and fragmentation conditions. Examples of optimized conditions for several ribonucleosides are listed in Table 1. These values will be used in Step 52 to set up the dynamic multiple reaction monitoring (DMRM) table.

**Troubleshooting (Table 2).**

**CRITICAL STEP** These conditions will vary significantly for different instruments and for different versions of the same instrument, so the conditions should be determined at least once for the user’s specific instrument.

### Determine the retention times of the modified ribonucleosides

Connect a Hypersil GOLD aQ 3 µm column (150 mm length × 2.1 mm inner diameter, 120 Å pore size 3 µm particle size; the UPLC version with 1.9 µm particle size also works well but other chromatographic parameters will differ) to the QQQ mass spectrometer system and equilibrate the column to 36 °C with 0.1% formic acid in HPLC-grade water at a flow rate of 0.4 ml/min for at least 20 min.

Dilute stocks of standard ribonucleosides in RNase-free water to give 0.1–1 ng/µl solutions, transfer each sample to a 100 µl vial insert tube, place the insert tube into a 2-ml screw top vial and cap each vial.

Inject the ribonucleoside standard onto the LC-MS system and note the retention time using the solvent gradient described in the following table. Examples of HPLC retention times for several ribonucleosides are listed in Table 1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Gradienta (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–6</td>
<td>0</td>
</tr>
<tr>
<td>6–7.65</td>
<td>0–1</td>
</tr>
<tr>
<td>7.65–9.35</td>
<td>1–6</td>
</tr>
<tr>
<td>9.35–10</td>
<td>6</td>
</tr>
<tr>
<td>10–12</td>
<td>6–50</td>
</tr>
</tbody>
</table>

---

Su et al. Page 14

Nat Protoc. Author manuscript; available in PMC 2015 February 02.
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Gradient&lt;sup&gt;a&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12–14</td>
<td>50–75</td>
</tr>
<tr>
<td>14–17</td>
<td>75</td>
</tr>
<tr>
<td>17–17.50</td>
<td>75–0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percent acetonitrile in 0.1% formic acid (vol/vol); flow rate 0.4 ml/min; column temperature 36 °C.

Quantification of ribonucleosides by LC-MS/MS

52] Create a run method in the Agilent acquisition software. For the LC parameters, use the values in step 49 for flow rate and step 51 for gradient parameters to set up the run gradient table. For mass spectrometer parameters, use the values from Mass Spectrometer Setup. For DMRM set up, use the values determined in Step 48 to set up the transitions, retention time and delta time for nucleotides; see the examples in Table 1.

53] Equilibrate the UPLC-coupled QQQ system as described in Step 49.

54] Create a work list in the MassHunter acquisition software, including the sample positions in the autosampler, the method that was set up in step 52, and the volume to inject for each sample.

55] Place the sample vials prepared in Step 45 in the autosampler positions noted in the work list from Step 54.

56] Run the work list. The total ion chromatogram of a typical run of digested yeast total tRNA is shown in Figure 5. NOTE: to prevent large variances in data, it is best to analyze all samples of the same experiment including control samples in the same run.

Data analysis

57] For each sample run, a sample data file will be generated by the acquisition software. Open this data with the Agilent Qualitative Analysis software, which is included with Agilent instruments and get the raw data for each sample. The TIC chromatogram is displayed by default. Right click on the sample file name and select “extract chromatogram” and then select MRM to extract all MRM peaks. The software will do an automatic integration of the peaks. The user will then need to manually check all peaks to make sure the auto-integration generated peaks are correct. If they are not, the user will have to implement a manual integration. Instructions for manual integration can be found in the software help files.

58] After auto and manual integration open the “compound list” table. This table contains all the information of every compound found during the run including the MRM transition, retention time, peak width, peak height and peak area. Right click on the table and select “export”, then select file format as “xls” and file content as “every row”. Thus the data will be exported into an Excel spreadsheet. Troubleshooting (Table 2).
59] Normalize the signal intensity for each ribonucleoside by dividing the raw peak area for the ribonucleoside by the UV absorbance (in-line detector) peak areas for one or more canonical ribonucleosides. This normalization adjusts for variations in total tRNA in the sample. **Optional:** the signal intensity of each ribonucleoside can be further normalized against that of the internal standard ([15N]5-deoxyadenosine) from the same HPLC run to adjust for day-to-day fluctuation in MS sensitivity.

60] Calculate treatment-induced changes in the relative quantities of modified ribonucleosides as fold-change of normalized ribonucleoside levels relative to the normalized levels in the reference sample (e.g., untreated cells). Optional: for multiple treatment conditions and dozens of modified ribonucleosides, use multivariate statistical analysis to define patterns in the fold-change data. An example of hierarchical clustering analysis is illustrated in the heat map shown in Figure 3.

**TIMING**

Steps 1–2, cell culture and harvesting: 3–6 h for *E. coli*, 1 d for *S. cerevisiae* and 3–4 d for mammalian cells

Steps 3–17, cell lysis and total RNA extraction: 1–2 h

Steps 18–37, total tRNA isolation: 2–3 h

Steps 38–45, hydrolysis of total tRNA: 4–5 h

Steps 46–51, LC-MS optimization: 1–2 d

Steps 52–56, quantification of ribonucleosides: 1 h to 1 d depending on the number of samples

Steps 57–60, data analysis: 3–5 h

**ANTICIPATED RESULTS**

The protocol described here provides a means to detect significant changes in the relative quantities of tRNA modifications in cells subjected to stimuli and exposed to different agents. Practitioners can anticipate detection and quantification of most modified ribonucleosides in approximately 0.5–10 µg of tRNA from any source, with *m/z* and retention time values used to identify those modified ribonucleosides of unknown identity (*i.e.*, molecules shown to possess a ribose or 2'-O-methyl ribose moiety). To demonstrate the power of the method, we examined the tRNA modifications of two *S. cerevisiae* strains, wild-type and *trm9Δ*, where the tRNA methyltransferase 9 (*TRM9*) gene is deleted. Trm9 is required for the formation of 5-methoxycarbonylmethyl-2'-O-methyluridine (mcm5U) and mcm5s2U at wobble positions of certain tRNAs. Using the workflow described here, we analyzed the ribonucleosides of tRNAs from both the wild-type and *trm9Δ* cells. A comparison of the two sets of data revealed that the relative quantities of most modified ribonucleosides remained unchanged, except for mcm5U and mcm5s2U (Figure 3, structures...
in Figure 1). As expected, in wild-type yeast, both mcm5U and mcm5s2U are present, whereas the two are not detectable in trm9Δ cells (Figure 3).

The method is highly precise, with 3.1% intra-day variance in average signal intensity and 12.1% inter-day variance in average fold-change values for each ribonucleoside in tRNA from yeast cells (statistics determined from 294 analyses in three biological replicates over several weeks).15 These data point to the importance of reducing variance by performing critical comparative analyses on the same day.

ACKNOWLEDGMENTS

We thank Drs. Koli Taghizadeh and John Wishnok for assistance with chromatography and mass spectrometry, which were performed in the Bioanalytical Facilities Core of the MIT Center for Environmental Health Sciences. Financial support was provided by the National Institute of Environmental Health Sciences (ES002109, ES015037 and ES017010), the MIT Westaway Fund, the Merck–MIT Graduate Student Fellowship (C.T.Y.C.), the David H. Koch Graduate Cancer Research Fellowship (C.G.), the Howard Hughes Medical Institute International Student Research Fellowship (C.G.) and the Singapore-MIT Alliance for Research and Technology.

REFERENCES


Nat Protoc. Author manuscript; available in PMC 2015 February 02.
31. Dewe JM, et al. The yeast rapid tRNA decay pathway competes with elongation factor 1A for substrate tRNAs and acts on tRNAs lacking one or more of several modifications. RNA. 2012; 18:1886–1896. [PubMed: 22895820]
Figure 1.
Chemical structures of modified ribonucleosides described in this protocol.
Figure 2.
Workflow for the quantitative analysis of modified ribonucleosides in tRNA. The blue highlighted boxes represent the steps described in this protocol.
Figure 3.
Analysis of LC-MS data from modified ribonucleoside studies. (A) Hierarchical cluster analysis of toxicant-induced changes in tRNA modification spectra in wild-type yeast exposed for one hour to concentrations of methylmethansulfonate (MMS), H\textsubscript{2}O\textsubscript{2}, NaOCl, and NaAsO\textsubscript{2} producing 20%, 50%, and 80% cytotoxicity; ribonucleoside structures are shown in Figure 1. The red color represents increase in fold change while green color represents decrease in fold change. (Reproduced from Chan et al. PLoS Genetics 6(12): e1001247, 2010) (B) Selected ion chromatograms for the Trm9-dependent ribonucleosides mcm\textsuperscript{5}U, and mcm\textsuperscript{5}s\textsuperscript{2}U in \textit{S. cerevisiae}. The MRM transitions used in identification and quantification of the ribonucleosides (examples in Table 1) are illustrated for the ribonucleoside structures. The numbers on the structures are the mass-to-charge ratios of the fragments, which are detected by the specific MRM transitions.
Figure 4.
RNA quality control analysis on an Agilent Bioanalyzer. Total human TK6 cell RNA (red line) was resolved on a RNA Pico Chip in an Agilent 2100 Bioanalyzer. Size markers are noted in blue.
Figure 5.
Total ion chromatogram from LC-MS/MS analysis of yeast tRNA ribonucleosides, to demonstrate resolution of modified ribonucleosides by reversed-phase HPLC. (Reproduced from Chan et al. PLoS Genetics 6(12): e1001247, 2010)
## TABLE 1

Dynamic MRM parameters for ribonucleosides based on Optimizer results.

<table>
<thead>
<tr>
<th>Name</th>
<th>Precursor Ion (m/z)</th>
<th>Product Ion (m/z)</th>
<th>Fragment (V)</th>
<th>Collision (V)</th>
<th>Ret. Time (min)</th>
<th>Ret. Window (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{15})N(_2)-dA</td>
<td>257</td>
<td>141</td>
<td>90</td>
<td>10</td>
<td>5.4</td>
<td>2</td>
</tr>
<tr>
<td>ac(_2^)C</td>
<td>286</td>
<td>154</td>
<td>80</td>
<td>6</td>
<td>9.5</td>
<td>2</td>
</tr>
<tr>
<td>Am</td>
<td>282</td>
<td>136</td>
<td>100</td>
<td>15</td>
<td>7.9</td>
<td>3</td>
</tr>
<tr>
<td>Cm</td>
<td>258</td>
<td>112</td>
<td>80</td>
<td>8</td>
<td>3.45</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>247</td>
<td>115</td>
<td>80</td>
<td>5</td>
<td>1.45</td>
<td>2</td>
</tr>
<tr>
<td>Gm</td>
<td>298</td>
<td>152</td>
<td>80</td>
<td>7</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>I</td>
<td>269</td>
<td>137</td>
<td>80</td>
<td>10</td>
<td>3.9</td>
<td>3</td>
</tr>
<tr>
<td>i(^6)A</td>
<td>336</td>
<td>204</td>
<td>100</td>
<td>17</td>
<td>12.6</td>
<td>2</td>
</tr>
<tr>
<td>m(^1)A</td>
<td>282</td>
<td>150</td>
<td>100</td>
<td>16</td>
<td>2.4</td>
<td>2</td>
</tr>
<tr>
<td>m(^1)G, m(^2)G</td>
<td>298</td>
<td>166</td>
<td>90</td>
<td>10</td>
<td>9.05</td>
<td>3</td>
</tr>
<tr>
<td>m(^1)I</td>
<td>283</td>
<td>151</td>
<td>80</td>
<td>10</td>
<td>8.5</td>
<td>2</td>
</tr>
<tr>
<td>m(^2)G</td>
<td>312</td>
<td>180</td>
<td>100</td>
<td>8</td>
<td>10.9</td>
<td>2</td>
</tr>
<tr>
<td>m(^2)A</td>
<td>282</td>
<td>150</td>
<td>100</td>
<td>16</td>
<td>9.5</td>
<td>2</td>
</tr>
<tr>
<td>m(^3)C, m(^3)C</td>
<td>258</td>
<td>126</td>
<td>80</td>
<td>8</td>
<td>2.2</td>
<td>2</td>
</tr>
<tr>
<td>m(^3)U</td>
<td>299</td>
<td>127</td>
<td>80</td>
<td>7</td>
<td>4.5</td>
<td>2</td>
</tr>
<tr>
<td>m(^5)Um</td>
<td>273</td>
<td>127</td>
<td>100</td>
<td>10</td>
<td>10.3</td>
<td>2</td>
</tr>
<tr>
<td>m(^5)A</td>
<td>296</td>
<td>164</td>
<td>100</td>
<td>16</td>
<td>11.5</td>
<td>2</td>
</tr>
<tr>
<td>m(^5)G</td>
<td>298</td>
<td>166</td>
<td>90</td>
<td>10</td>
<td>3.5</td>
<td>2</td>
</tr>
<tr>
<td>mcm(^5)(^2)U</td>
<td>333</td>
<td>201</td>
<td>100</td>
<td>10</td>
<td>11.1</td>
<td>2</td>
</tr>
<tr>
<td>mcm(^5)(^3)U</td>
<td>333</td>
<td>169</td>
<td>100</td>
<td>12</td>
<td>11.1</td>
<td>2</td>
</tr>
<tr>
<td>mcm(^5)U</td>
<td>317.2</td>
<td>185.1</td>
<td>90</td>
<td>8</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>mcm(^5)U</td>
<td>317.2</td>
<td>153.1</td>
<td>90</td>
<td>8</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>mcm(^5)Um</td>
<td>331.2</td>
<td>185.1</td>
<td>90</td>
<td>8</td>
<td>11.3</td>
<td>2</td>
</tr>
<tr>
<td>mcm(^5)Um</td>
<td>331.2</td>
<td>153.1</td>
<td>90</td>
<td>8</td>
<td>11.3</td>
<td>2</td>
</tr>
<tr>
<td>ncm(^5)U</td>
<td>302</td>
<td>170</td>
<td>100</td>
<td>8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ncm(^5)U</td>
<td>302</td>
<td>153</td>
<td>100</td>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>rN Name</td>
<td>Precursor Ion (m/z)</td>
<td>Product Ion (m/z)</td>
<td>Fragment (V)</td>
<td>Collision (V)</td>
<td>Ret. Time (min)</td>
<td>Ret. Window (min)</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------</td>
<td>-------------------</td>
<td>--------------</td>
<td>---------------</td>
<td>-----------------</td>
<td>------------------</td>
</tr>
<tr>
<td>r6A</td>
<td>413</td>
<td>281</td>
<td>100</td>
<td>8</td>
<td>12.0</td>
<td>2</td>
</tr>
<tr>
<td>Um</td>
<td>239</td>
<td>113</td>
<td>80</td>
<td>7</td>
<td>5.8</td>
<td>2</td>
</tr>
<tr>
<td>Ψ</td>
<td>245</td>
<td>125</td>
<td>80</td>
<td>10</td>
<td>1.5</td>
<td>2</td>
</tr>
</tbody>
</table>

1. The full name for each ribonucleoside is noted in Figure 5.

2. All ions were detected in positive ion mode. Cell acceleration voltage was set to 7 for all transitions.
## TABLE 2

**Troubleshooting table**

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason(s)</th>
<th>Solution(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step 15</strong></td>
<td>$\text{OD}<em>{260}/\text{OD}</em>{280}$ is less than 1.9</td>
<td>DNA contamination during RNA isolation</td>
<td>Treat the RNA samples with DNase I according to manufacturer’s directions. This is typically a 1-hr incubation at ambient temperature or 37 °C.</td>
</tr>
<tr>
<td><strong>Step 16</strong></td>
<td>Yield lower than expected and/or appearance of a wide peak of small RNAs</td>
<td>RNA degradation</td>
<td>Discard the sample and re-isolate total RNA from fresh cells</td>
</tr>
<tr>
<td><strong>Step 48</strong></td>
<td>Weak signal for ribonucleosides during optimization</td>
<td>The concentration of the nucleoside is below the detection limit</td>
<td>Increase the concentration of the nucleoside five times and repeat the optimization process</td>
</tr>
<tr>
<td><strong>Step 58</strong></td>
<td>Large shifts in retention time of ribonucleosides form experiment to experiment</td>
<td>HPLC pump malfunction due to leaks or other problems</td>
<td>Flush the HPLC pump and check the pressure to identify the leaks; repair the pump</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Altered buffer conditions</td>
<td>Use freshly prepared buffers Before the run, check sample tubes for air bubbles and remove bubbles by flicking the bottom of the tube; degas the HPLC solvents by sonication.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Possible air bubbles in the solvent or sample</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insufficient time to equilibrate the column between consecutive runs.</td>
<td>Wash the column with mobile phase A for a longer time and at a higher flow rate.</td>
</tr>
</tbody>
</table>