

# Isolation of a site-specifically modified RNA from an unmodified transcript

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## ABSTRACT

**Natural RNAs contain many base modifications that have specific biological functions. The ability to functionally dissect individual modifications is facilitated by the identification and cloning of enzymes responsible for these modifications, but is hindered by the difficulty of isolating site-specifically modified RNAs away from unmodified transcripts. Using the m1G37 and m1A58 methyl modifications of tRNA as two examples, we demonstrate that non-pairing base modifications protect RNAs against the DNA-directed RNase H cleavage. This provides a new approach to obtain homogeneous RNAs with site-specific base modifications that are suitable for biochemical and functional studies.**

## INTRODUCTION

RNA molecules undergo extensive post-transcriptional modifications that are important for their biological activities. A major category of post-transcriptional modifications is 2'-O-ribose methylation of the backbone, which occurs frequently in ribosomal RNA and often at positions that are conserved in evolution and essential for translation (1). The other category of post-transcriptional modifications involves nucleotide bases, which are predominantly found in tRNAs. To date, the number of different types of base modifications identified in tRNAs exceeds 100 (2). These modifications enrich the chemical properties of nucleotide bases in ways that are similar to those of amino acid side-chains, such as aliphatic, aromatic, polar and charged features (3). The diversity of base modifications suggests that all organisms, bacteria, eukarya and archaea, have a large investment in the metabolic and energy requirements for tRNA modifications (4). Although many of the tRNA base modifications are not understood at the functional level, some have well-defined roles in translation and decoding of genomes. For example, modifications that occur at the wobble position of the tRNA anticodons,

and those 3' adjacent to the anticodon make a large contribution to translational efficiency and accuracy (5–7), and help to maintain a proper reading frame (8). The existence of these modifications, diverse among different tRNA anticodon sequences, demonstrates cellular dedication to the specificity of decoding.

Studies of base modifications have benefited from identification and cloning of genes responsible for modifications. For example, the tRNA base modification m1G37 is catalyzed by the enzyme tRNA(m1G37) methyl transferase, using S-adenosyl methionine as a methyl donor to the N1 position of G37 (9). This modification is important for maintaining reading frame fidelity during decoding (10–12). Genetic studies have identified the *trmD* gene of bacteria (13), and *trm5* gene of eukarya and archaea (14,15) as responsible for encoding the enzyme tRNA(m1G37) methyl transferase. Representative *trmD* and *trm5* genes have been cloned and expressed in *Escherichia coli* to yield active enzymes (15,16), using tRNA transcripts lacking any modifications as substrates.

The ability to generate tRNA molecules containing a single modification, introduced by the reaction of a well-defined enzyme, offers new opportunities to dissect the function of the specific modification. However, a major obstacle remains, which is that the modification reaction is usually not stoichiometric. For example, the m1G37 modification on a transcript of tRNA<sup>Cys</sup>, as generated by reactions using purified *E. coli* TrmD or an archaeal Trm5, is typically 30–50%, even with excess of enzyme (15,17). The reason for the incomplete reaction is unclear, but may be due to loss of enzyme activity in some cases, or due to partial folding of the tRNA transcript in other cases that prevent a proper anticodon loop structure for modification. The incomplete reaction yields mixtures of both modified and unmodified transcripts, which hinders rigorous interpretation of biochemical studies. Thus, it is highly desirable to have a method to separate the modified transcript from the unmodified transcript. Because the two types of transcripts differ by a single base modification, our experience showed that conventional separation by denaturing gel electrophoresis or by liquid chromatography is difficult.

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Here we describe a simple method of separation that can be easily developed in any laboratory without sophisticated instruments. This method is applicable to base modifications that interfere with Watson–Crick base pairing (e.g. m1G37), which account for 40–50% of the more than 100 base modifications in databases. We demonstrate that such a base modification confers resistance to pairing with complementary oligonucleotides and thus resistance to RNase H, which cleaves the RNA strand of an RNA–DNA hybrid (18,19). In contrast, RNA with the unmodified base is susceptible to DNA hybridization and thus cleavage by RNase H. Previous studies have used a similar approach to identify sites of 2'-O-ribose methylation in RNA molecules (20). Because the RNase H-dependent purification is based on accessibility of the base modification to hybridization, we tested its application to both the m1G37 modification, located in the anticodon loop, and the m1A58 modification, located in the more complex tRNA tertiary core region. In both cases, we successfully purified the tRNA species that contained the single base modification away from the unmodified transcript, illustrating the general utility of this method.

## MATERIALS AND METHODS

The T7 transcripts of *Methanococcus jannaschii* tRNA<sup>Cys</sup> and tRNA<sup>Pro</sup> were made by *in vitro* transcription as described (21). The His-tagged enzymes *M.jannaschii* Trm5, *Thermus thermophilus* Trm1 and yeast Trm10, were purified by a metal affinity column, followed by monoS on an FPLC (15,22,23). Concentrations of tRNAs were determined by ultraviolet (UV) absorption, while those of enzymes were determined by the Bradford assay. Oligo #1 and oligo #2 were chemically synthesized by IDT (Integrated DNA Technologies, IA) and used directly without further purification. To perform the RNase H cleavage, a tRNA and its specific oligo (6 nmol each) were mixed in a volume of 8  $\mu$ l TE buffer [10 mM Tris–HCl (pH 8.0) and 1 mM EDTA], heated to 85°C and adjusted to the RNase H buffer and 0.1 U of RNase H (Promega) to a final volume of 10  $\mu$ l. The reaction was incubated at 37°C, while aliquots of 3  $\mu$ l were removed at 0, 20 and 40 min, and analyzed by gel electrophoresis on a 12% PAGE/7 M urea on a BioRad MINI PROTEAN three Gel system. The gel was run at 200 V in TBE for  $\sim$ 50 min until bromophenol blue ran to the bottom. The gel was stained by ethidium bromide and visualized by UV. For gel shift analysis, modified and unmodified *M.jannaschii* tRNA<sup>Cys</sup> was <sup>32</sup>P-labeled at the 3' end by the CCA-adding enzyme (24), purified through a Centricon-20 device (Princeton Separation), heat-denatured and annealed with 3  $\mu$ M oligo #1 in the RNase H buffer. The annealed reactions were adjusted to 1 $\times$  TBE, 5 mM MgCl<sub>2</sub>, 8% glycerol, 0.1% xylene cyanol and 0.1% bromophenol blue, and applied to a native 12% PAGE (1 $\times$  TBE and 10 mM MgCl<sub>2</sub>) and electrophoresed at 100 V for 90 min at room temperature. The gel was dried and analyzed by a phosphorimager.

## RESULTS AND DISCUSSION

### Experimental design

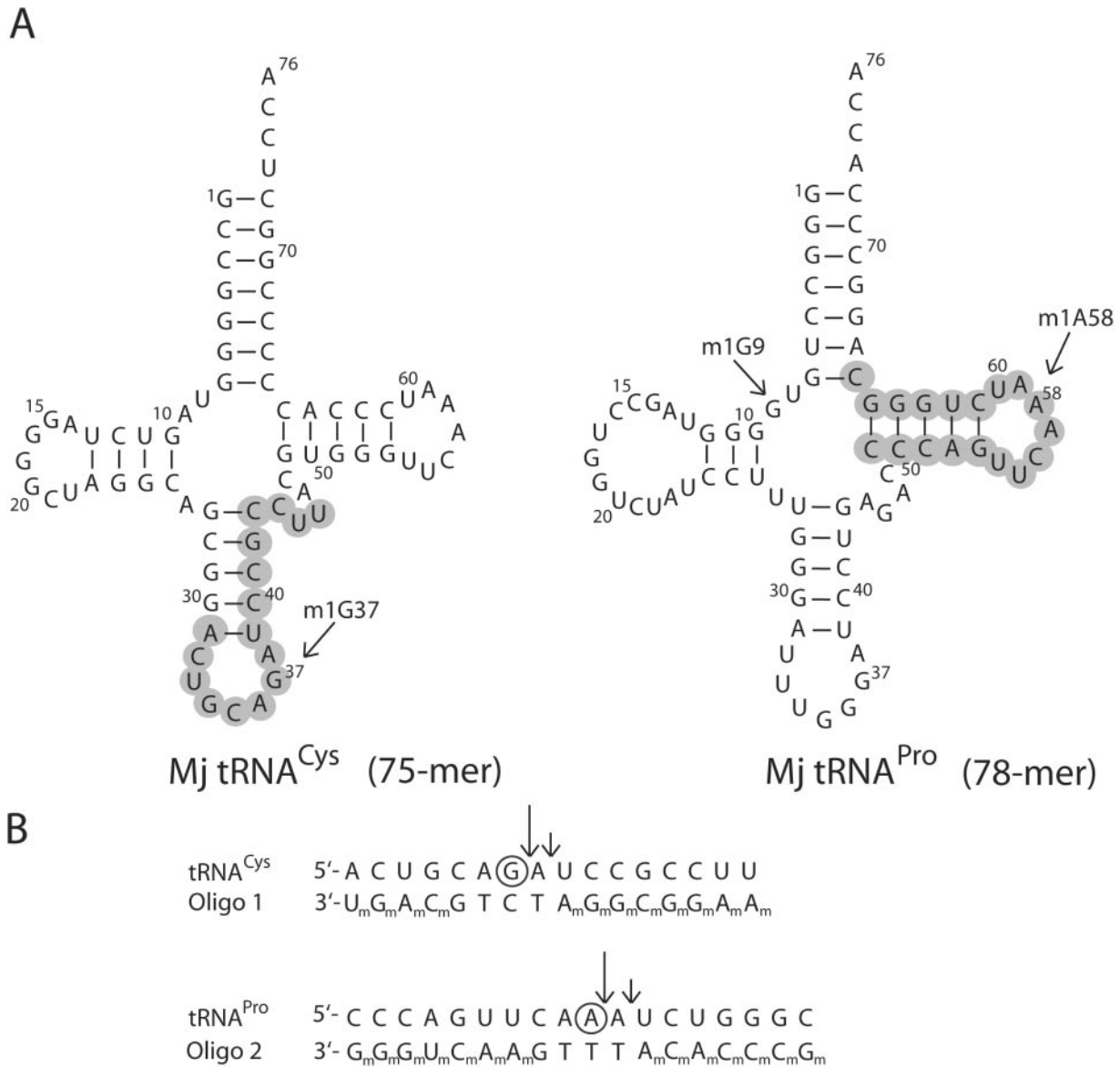
For the m1G37 modification, we used tRNA<sup>Cys</sup> as the substrate, while for the m1A58 modification, we used tRNA<sup>Pro</sup>

(Figure 1A). Both tRNAs were derived from the archaeon *M.jannaschii*, and both were GC rich relative to their bacterial counterparts such that they offered an opportunity to test sequences that were less accessible to complementary oligonucleotides. The two tRNAs were synthesized as unmodified transcripts by T7 RNA polymerase *in vitro*. Introduction of the m1G37 modification was achieved by the homologous *M.jannaschii* Trm5 (15), while that of the m1A58 modification was achieved by the *T.thermophilus* Trm1 (23). Both enzymes were expressed in *E.coli* and purified as His-tagged recombinant proteins. The extent of reaction by each enzyme was quantified by using [<sup>3</sup>H] AdoMet as the substrate and determined by measuring acid-precipitable counts on filter pads.

The oligonucleotides that targeted m1G37 and m1A58 were designed based on previous studies, which showed that the DNA-directed RNaseH cleavage of the RNA strand can be site-specific if the oligonucleotide consists of four deoxynucleotides flanked by 2'-O-methyl ribonucleotides on two sides (20,25,26). In this design, the cleavage occurred at one of two sites. The predominant site was the phosphodiester bond 5' to the RNA residue that is base paired with the 5'-most DNA residue of the chimera (e.g. the large arrows in Figure 1B) (20), while the minor site was the phosphodiester bond 3' to the same RNA residue (the small arrows) (25). Either scenario would lead to cleavage of the RNA strand. Thus, oligo #1 was designed to complement tRNA<sup>Cys</sup> from A31 to U46, encompassing G37 in the anticodon loop, whereas oligo #2 was designed to complement tRNA<sup>Pro</sup> from C49 to C66, encompassing A58 in the T loop (Figure 1B), where it usually forms a reverse Hoogsteen base pair with U54. In both designs, the primary cleavage on tRNA would be 3' to the site of base modification. Oligos #1 and #2 were chemically synthesized with a 2'-O-methyl backbone, except for four deoxyribose nucleotides that complemented the region of the respective modification site.

### Base modification protects RNA from RNase H

The ability of m1G37 and m1A58 to protect tRNA against RNase H was examined (Figure 2). The Trm5-modified tRNA<sup>Cys</sup> transcript was mixed with oligo #1 at a 1:1 molar ratio, and the mixture was heat-denatured and annealed in the presence of the RNase H buffer. *E.coli* RNase H was added to the annealed hybrid and aliquots at various incubation times (0, 20 and 40 min) were quenched with a dye solution containing 8 M urea. In parallel, an unmodified tRNA<sup>Cys</sup> transcript (without the Trm5 reaction) was treated similarly to provide a comparison. Cleavage of tRNAs was analyzed by a denaturing 12% PAGE/7 M urea gel on a small apparatus and stained by ethidium bromide. While the unmodified transcript was completely cleaved in 20 min, yielding two fragments of similar lengths (37 and 38mers), a significant fraction of the modified transcript showed resistance to RNase H up to 40 min (Figure 2A). This fraction was  $\sim$ 40% in 40 min, which was similar to that determined from the plateau level of the m1G37 methylation reaction (37%). The cleaved fragments appeared <50%, which was accounted for by the shorter sizes the fragments that were weakly stained by ethidium bromide as evidenced in reactions of both unmodified and modified transcripts. The cleavage-resistant fraction remained constant in 40–60 min, even with addition of more RNase H (data not



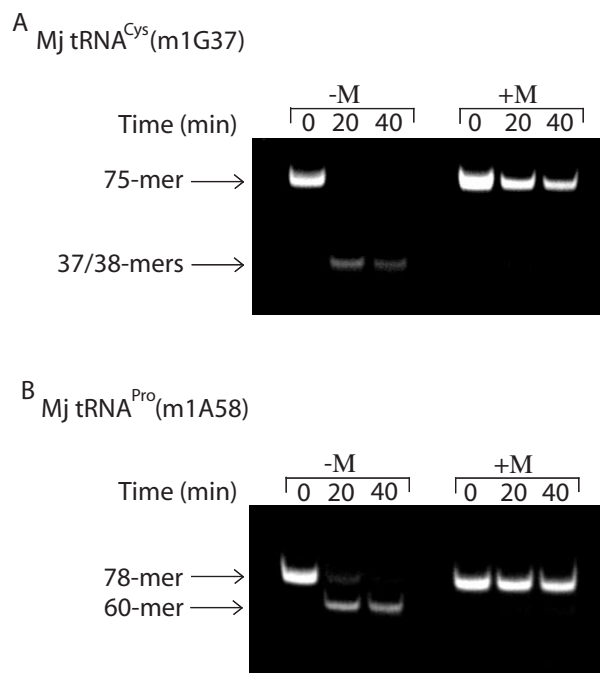
**Figure 1.** (A) Sequence and cloverleaf structure of *M. jannaschii* tRNA<sup>Cys</sup> and tRNA<sup>Pro</sup>, where the m1G37, m1A58 and m1G9 modifications are indicated. Shaded residues are complements of oligo #1 and oligo #2. (B) Schematic representation of RNase H cleavage sites. The top strand is the target sequence in tRNA<sup>Cys</sup> and tRNA<sup>Pro</sup>, respectively, hybridized to oligo #1 and oligo #2. The 2'-O-methyl backbone modification is indicated by a subscripted 'm' preceding the nucleotide base, while positions of m1G37 and m1A58 are shown by circles. The large arrows indicate the predicted primary cleavage sites, whereas small arrows indicate the secondary cleavage sites.

shown), suggesting that the cleavage was complete and that little unmodified transcript was left uncleaved.

Similarly, the Trm1-modified tRNA<sup>Pro</sup> transcript was hybridized to oligo #2 and subjected to the RNase H cleavage analysis (Figure 2B). While the unmodified transcript was cleaved within 20 min, the modified transcript remained largely intact. The cleavage of the unmodified transcript generated a 60mer fragment of the expected size (Figure 2B) and a smaller 18mer, which migrated off the gel, leaving ~5% full-length tRNA that was cleaved upon further incubation (data not shown). The cleavage-resistant fraction of the modified transcript was ~90%, similar to values (82%) measured by the plateau methylation of the m1A58 reaction, indicating a more efficient methylation reaction than the m1G37 reaction. In

both the Trm5 and Trm1 reactions, the corroboration between the cleavage-resistant fraction and the methylation data was confirmed over 5–8 independent experiments.

The RNase H cleavage reaction was also tested with the m1G9 modification in *M. jannaschii* tRNA<sup>Pro</sup>, which was at the junction between the acceptor and D stems (Figure 1) and was introduced by the Trm10-catalyzed reaction (22). As expected, the control transcript was quantitatively cleaved to give a 69mer fragment, whereas the modified transcript was only partially cleaved (data not shown). However, at longer incubation times both the 69mer fragment and the full-length modified transcript were cleaved at a secondary site, which exhibited partial complementarity to the central portion of the targeting oligonucleotide. This example emphasizes that

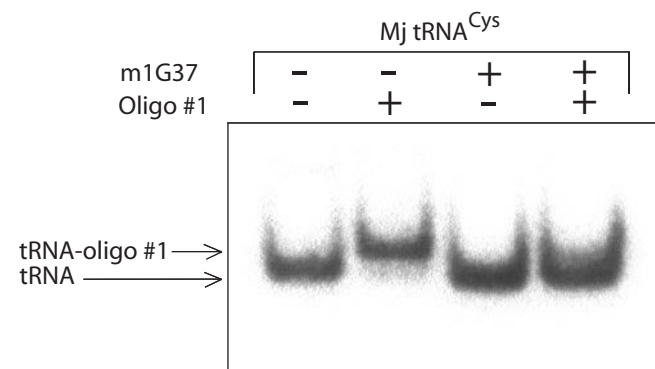


**Figure 2.** Protection of tRNA against RNase H cleavage in (A) by the m1G37 modification in the transcript of *M. jannaschii* tRNA<sup>Cys</sup> and in (B) by the m1A58 modification in the transcript of *M. jannaschii* tRNA<sup>Pro</sup>. Unmodified transcripts are indicated by '-M', while modified transcripts are indicated by '+M'. The sizes of full-length and cleaved fragments of tRNA<sup>Cys</sup> and tRNA<sup>Pro</sup> are indicated on the side.

the specificity of cleavage requires that the site of modification does not resemble other sequences in the same RNA.

### Base modifications prevent RNA hybridization to oligonucleotides

The ability of a base modification to protect tRNA against RNase H cleavage could arise from inhibition of the cleavage reaction by the non-hydrogen bonding base, or from failure of the oligonucleotide to hybridize to the modified RNA, thus preventing RNase H cleavage. To distinguish between these two possibilities, complex formation between tRNA<sup>Cys</sup> and oligo #1 was examined by gel electrophoresis under native conditions, containing 5 mM MgCl<sub>2</sub> (27). Here the m1G37-modified tRNA was first purified away from the unmodified transcript by the RNase H cleavage reaction described above. The modified and unmodified transcripts were then labeled with <sup>32</sup>P by the CCA-adding enzyme, heat-denatured and annealed with a molar excess of oligo #1, then analyzed by gel electrophoresis (Figure 3). Clearly, in the reaction with the unmodified tRNA, almost complete shift of the tRNA by the presence of oligo #1 was observed (~95%). In contrast, in the reaction with the modified tRNA, only a small amount of the shift was observed (~5%), suggesting that either the hybrid was unstable during the gel electrophoresis condition or that the modified tRNA was not accessible to hybridization. A separate experiment using <sup>32</sup>P-labeled oligo #1 confirmed that the oligo was unable to form a stable hybrid with the modified transcript (data not shown). These experiments



**Figure 3.** Phosphorimager analysis of hybridization of oligo #1 to transcripts of *M. jannaschii* tRNA<sup>Cys</sup>. The modified transcript was purified using the RNase H cleavage reaction. Migration positions of the unbound tRNA and tRNA-oligo hybrid are indicated by arrows.

demonstrated that a single base modification in a tRNA effectively destabilized or prevented hybridization to its complementary 17mer oligonucleotide. Earlier studies have also shown that a single mismatch eliminates hybridization of 17mer primers to their target sequences (28,29).

The inability of the modified tRNA to form a stable hybrid with the oligonucleotide provides a relevant rationale for why the tRNA was resistant to RNase H cleavage. Based on the analytical gel shown in Figure 3, the modified tRNA appeared to migrate slightly faster than the unmodified tRNA, suggesting the possibility of a conformational change. Recent structural analysis of an m1G37-modified anticodon loop has shown that the modification indeed rigidifies the RNA by reducing molecular dynamics (30). This conformational rigidity might further deter hybridization with oligo #1 and explain the fractional shift in mobility of the modified tRNA.

Although Figure 3 illustrates the clear distinction between the modified and unmodified tRNAs in their hybridization behaviors, it should be emphasized that hybridization alone is not sufficient to completely separate the two types of tRNA. For one reason, the mobility shift of the hybrid is rather small under the native gel conditions (Figure 3), suggesting that separation of the unmodified transcript in the hybrid from modified transcript by gel shift would be difficult, particularly on a preparative gel with a scaled-up reaction. Second, while it might be possible to develop a pull-down method to remove the unmodified transcript by using a biotin-tagged oligonucleotide to trap the hybrid to streptavidin beads, this method will also remove some of the modified transcript. It is evident from analysis of Figure 3 that the modified transcript showed fractional hybridization to oligo #1.

The RNase H cleavage reaction following hybridization provides the necessary step to completely separate the modified from the unmodified transcript. Even if the modified transcript has the ability to form a hybrid, regardless of the stability, this hybrid is not a substrate for the RNase H cleavage activity. In the recent crystal structure of the *Bacillus halodurans* RNase H bound to an RNA/DNA hybrid (19), the substrate specificity depends on direct interactions between the enzyme active site that contacts the RNA strand of the RNA/DNA hybrid. This enzyme uses the two-metal-ion catalysis for the cleavage reaction (31). Two of the catalytic carboxylates that coordinate the metal ions make hydrogen-bonds with two

2'-OH groups immediately 5'-to the scissile phosphate, whereas two other catalytic residues contact the two 2'-OH groups 3' to the scissile phosphate. These contacts are made by conserved residues of the active site and explain why cleavage requires a minimum of four consecutive riboses, two on each side of the scissile bond (32). Notably, our design of oligonucleotides placed the modification in the middle of the 4 bp of the RNA/DNA hybrid (Figure 1B). This is to eliminate the base pair in the middle of the hybrid and to destroy the required continuity of four 2'-OH groups along the active site groove. The mis-alignment of the 2'-OH groups would perturb the active site, thus providing a clear rationale for the lack of RNase H cleavage activity.

The method developed here allows a general RNA transcript containing a site-specific base modification to be gel purified and separated from the unmodified transcript, which is cleaved. The purified RNA is now suitable for investigation *in vitro* to determine the function of the base modification. At present, the only other alternative is to prepare a site-specifically modified RNA by chemical synthesis, which is limited by the type of modification (30), the high cost of synthesis, and the length of RNA. A compromise to the high cost and length limitations is to synthesize smaller RNA fragments containing modifications and use RNA or DNA ligase to join them to unmodified RNAs (33,34). However, successful ligation requires extensive investigation of conditions and may not be efficient (Z. Li and Y.-M. Hou, unpublished data). These drawbacks are not inherent to our method, which is rapid and robust, versatile for modifications in highly structure RNA, and has no length limitation. The only equipment necessary for the method is a mini-gel apparatus that provides separation of cleaved from uncleaved RNA species. In scaled-up reactions, we routinely apply 5–10 nmols of RNA into one lane of the mini-gel apparatus and use UV shadowing to identify RNA bands. The modified RNA can then be eluted from gel slices. The method requires custom synthesis of complementary 2'-O-methyl oligonucleotide, which is facile and affordable by current technology.

Despite the multiple advantages, some critical points need to be considered for application of this method. First, it requires the availability of the requisite modification enzyme and a base modification that disrupts conventional base pairing. Because each modification is distinct in chemical nature and its ability to disrupt base pairing, the condition of hybridization and RNase H cleavage reaction needs to be identified for each. This is evident in Figure 2, where the m1G37 modification catalyzed by the Trm5 enzyme is less efficient than the m1A58 modification catalyzed by the Trm1 enzyme. Also, the m1G37-modified tRNA requires longer time (40–60 min) to be cleared of the unmodified transcript than the m1A58-modified tRNA (20–40 min). Second, the design of oligonucleotides should take into account the position of the modified base and length of flanking sequences. The general principle is to design oligonucleotides that will place the modified base in the middle of the four RNA/DNA hybrid base pairs as demonstrated here. The flanking sequences should be such that oligonucleotides can form stable hybrids with unmodified transcripts at 37°C. This can be determined by a heat-cool process, followed by gel shift analysis. We suggest that 17mer is sufficient for GC rich sequences, such as *M.jannaschii* tRNA<sup>Cys</sup> and tRNA<sup>Pro</sup>, but that longer

oligonucleotides might be necessary for AU rich sequences. Finally, the sequence of oligonucleotides should avoid ambiguity, such that hybridization can take place at only one site. However, this may not be possible for all modifications, as demonstrated by the example of the m1G9 modification.

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