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A map of 5-methylcytosine residues in *Trypanosoma brucei* tRNA revealed by sodium bisulfite sequencing

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Abstract

In protozoan parasites, there is little information on the presence of covalent RNA modifications which comprise the epitranscriptome. Therefore, we determined if *T. brucei* $tRNA^{Asp(GUC)}$, $tRNA^{Gly(GCC)}$, $tRNA^{Val(AAC)}$, and $tRNA^{Tyr(GUA)}$ contain 5-methylcytosines via RNA bisulfite sequencing. Most tRNAs examined have at least one 5-methylcytosine at the variable region-TψC junction. Only $tRNA^{Gly(GCC)}$ displayed methylation of C40 in the anticodon stem, and there was partial methylation at this site. There is no evidence for methylation of C38 in the anticodon loop in the tRNAs analyzed. Analysis of $tRNA^{Tyr(GUA)}$ demonstrates that both unspliced and spliced molecules contain C48 methylation, indicating tRNA cytosine methylation can precede tRNA splicing. Overall, our data indicate that *T. brucei* tRNAs contain 5-methylcytosine residues in some, but potentially not all standard eukaryotic positions. The levels of cytosine methylation of different *T. brucei* tRNAs vary, suggesting the presence of a mechanism for methylation control.

Keywords

tRNA; 5-methylcytosine; sodium bisulfite sequencing; Dnmt2; NSun2

Epigenetic information is heritable genetic information that is not based on DNA sequence alone. One of the main types of epigenetic information is the presence of methyl groups. Both protein and DNA are methylated, and these modifications can impact numerous processes including gene expression, molecular structure, and DNA repair. RNA can be chemically modified after transcription, and this finding has led to the realization that, in addition to an epigenome, organisms possess an epitranscriptome [1]. RNA methylation events are at the heart of the epitranscriptome. RNA molecules can be methylated on both the sugar and base, and the focus of this work is RNA cytosine base methylation. There are many different types of RNAs that can be modified by cytosine methylation. In *Escherichia*

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coli and archaebacteria, 16S and 23S rRNA are modified by cytosine base methylation, and human 28S rRNA is modified as well [2, 3]. In archaebacteria and eukaryotes, but apparently not bacteria, tRNAs are methylated in a tRNA-specific manner and hotspots include the anticodon loop, anticodon stem, and the variable region-TψC junction [4]. Recent reports also indicate that there are 5-methylcytosine residues in mRNA from the archaeon *Solfolobus solfataricus* and 8,495 5-methylcytosine residues in the HeLa cell mRNA transcriptome [2, 5]. In HeLa cells, the presence of mRNA harboring 5-methylcytosine is dependent upon an enzyme that methylates tRNA (NSun2), suggesting a link between mRNA and tRNA methylation [5].

We were interested in determining if RNA methylation pathways exist in *T. brucei* for two reasons. First, in *T. brucei*, regulation of gene expression occurs at the posttranscriptional level where RNA is a key player and has been a focus of numerous investigations. Thus, RNA modifications may be especially important with respect to gene regulation in this organism. Second, it has been demonstrated that the eukaryotic enzyme DNA methyltransferase 2 (Dnmt2) has the ability to methylate RNA, in particular tRNA [6]. This may be relevant to protozoan parasites, as DNA methyltransferase enzymes have been identified in several organisms including *Trypanosoma brucei* and *Entamoeba histolytica* [7, 8]. However, a role for DNA methylation in protozoan parasites is relatively undefined. For example, in *Entamoeba histolytica*, blocking DNA methylation with the inhibitor 5-azacytidine affects the expression of only a small subset of genes (~2%) [9]. Additionally, some protozoan parasites such as *Cryptosporidium parvum* and *Plasmodium falciparum* contain DNA methyltransferase homologues but lack detectable DNA methylation [7, 10], which suggests that protozoal DNA methyltransferases may modify another target such as RNA.

With respect to RNA targets, we started with cytosine base modification of *T. brucei* tRNAs, as tRNAs are the best described methylated RNAs in other organisms. Eukaryotic tRNAs are methylated at several defined locations [4]. The majority of eukaryotic tRNAs that have been examined are methylated at the junction between the variable region and TψC stem at one or more positions via NSun2 (Trm4a/b in *S. pombe*) [4]. NSun2 is able to methylate cytosine 40 (C40) in the anticodon stem in some eukaryotic tRNAs as well [11]. C38 in the anticodon loop is methylated by Dnmt2 (pmt1 in *S. pombe*) in *S. pombe*, *Drosophila melanogaster*, mice, and humans [5-7, 12-14]. Dnmt2 was originally identified as a DNA methyltransferase homologue, but only has weak enzymatic activity toward DNA both *in vitro* and *in vivo* and is thought to function primarily as a tRNA methyltransferase [6, 7]. Other targets for cytosine modification also exist. For example, cytosines 60-62 are methylated in *S. pombe* $tRNA^{Asp(GUC)}$, and methylation is abrogated in trm4a/trm4b double-knockout strain [12]. It is currently unclear if cytosine base modifications exist in *T. brucei* RNA.

To determine if 5-methylcytosines exist in *T. brucei* tRNA, we used a sodium bisulfite sequencing approach. In this approach, RNA is treated with sodium bisulfite thereby converting cytosines to uracil. These residues are then present as thymines following cDNA synthesis and PCR amplification. Importantly, 5-methylcytosine is not converted to thymine by bisulfite treatment, and thus is read as a cytosine after DNA sequencing. This approach

has been used heavily for the analysis of DNA methylation and has recently been modified for use with RNA molecules including tRNA and mRNA [2, 3, 5, 12-14]. Is it important to note that bases other than 5-methylcytosine can be resistant to bisulfite conversion [5]. The best described resistant base is 5-hydroxymethylcytosine, although the presence of 5-hydroxymethylcytosine in RNA has not been reported to our knowledge [2, 15]. For simplicity, we have used the term 5-methylcytosine to describe cytosines that are resistant to sodium bisulfite-mediated deamination, but cannot exclude the presence of other modified cytosine bases. For the sodium bisulfite sequencing reactions described below, we followed the protocol of Schaefer *et al*. [13]. RNA (1-2.5 μg) isolated from *T. brucei* strain 29-13 was treated with sodium bisulfite for 3-4 cycles, purified, and used as a template for cDNA synthesis. Stem loop primers were used for cDNA synthesis to lengthen the tRNA amplicons (Table I, Figure 1A), as these amplicons are short and can comigrate with PCR primer dimers. PCRs were performed using primers that bind to the deaminated versions of the cDNA (Table I, Figure 1B). PCR products for all tRNA targets were only detected when cDNA was made in the presence of reverse transcriptase, indicating sequencing results are from the tRNA itself and not the tRNA gene (Figure 1C). PCR products were inserted into pGEM-T Easy plasmids and sequenced. 20 clones or more were analyzed per experiment.

Successful sodium bisulfite sequencing is heavily dependent on robust deamination of non-modified cytosines to uracil, and a lack of cytosine deamination will result in a high rate of false positives. To determine the deamination rate of our procedure in regards to tRNA, we prepared *in vitro* synthesized $tRNA^{Gly(GCC)}$ by transcription of a $tRNA^{Gly(GCC)}$ PCR product with an engineered T7 promoter. The *in vitro* transcription reactions were performed in the absence of 5-methylcytosine to generate unmodified $tRNA^{Gly(GCC)}$. The unmodified tRNA was analyzed by sodium bisulfite sequencing as described above. Only 9 of the 320 cytosines analyzed were resistant to bisulfite conversion (Figure 2A). Thus, the deamination rate for this reaction is 97.2%, and the level of false positives is low at <3%. Our RNA deamination rate is similar to rates recently reported by Edelheit *et al*. [2]. Most positions containing cytosines exhibited 100% deamination in our experiments, and all positions containing cytosines had 90% or greater deamination. Based on the data above, we considered specific cytosines with >10% bisulfite resistance to be 5-methylcytosines.

To sequence native *T. brucei* tRNAs, we originally started with *T. brucei* total RNAs generated by Trizol extraction. However, our initial attempts with total RNA were largely unsuccessful, despite the presence of small RNAs in the sample (Supplemental Figure 1A). This was due to non-specific amplification using the AT-rich tRNA primers (data not shown). Thus, we enriched *T. brucei* total RNA for small RNAs using LiCl precipitation. Total RNA was incubated in the presence of LiCl, and large RNAs were collected via centrifugation. The supernatant was precipitated and analyzed by RNA bioanalysis (Supplemental Figure 1B and 1C). The supernatant was enriched for small RNAs less than 200 nucleotides, with a major peak ~75 nucleotides in size which is the typical size of a tRNA. Small RNA fractions were used for all downstream experiments.

We selected specific tRNAs for sodium bisulfite sequencing based on studies using sodium bisulfite sequencing in other organisms [5, 11-14]. The tRNAs analyzed in this study were $tRNA^{Asp(GUC)}$, $tRNA^{Gly(GCC)}$, $tRNA^{Val(AAC)}$, and $tRNA^{Tyr(GUA)}$. The sequencing data are

presented as a list of sequences in Figures 2B-F. In addition, the positions of 5-methylcytosines are mapped to predicted secondary structures of the tRNAs using tRNAScan-SE (Figures 2G-K) [16]. Based on our sodium bisulfite sequencing data, $tRNA^{Gly(GCC)}$ showed robust methylation of three cytosines at the junction between the variable region and TψC stem, and partial methylation (29%) at C40 in the anticodon stem. There were no 5-methylcytosines present at C38 in the anticodon loop or at other positions in this tRNA. *T. brucei* $tRNA^{Asp(GUC)}$ showed robust methylation at the variable region-TψC junction at C47, C48, and C49. There was also weak evidence for the presence of 5-methylcytosine at C63 in this tRNA. There were no additional 5-methylcytosines found at other positions in *T. brucei* $tRNA^{Asp(GUC)}$, including C38 in the anticodon loop or C40 in the anticodon stem. For *T. brucei* $tRNA^{Val(AAC)}$, we observed robust methylation of C48 at the variable region-TψC junction, but not C50 in the TψC stem. There were no other 5-methylcytosines found in *T. brucei* $tRNA^{Val(AAC)}$, including C38 in the anticodon loop.

In *T. brucei*, only one tRNA requires splicing as part of the maturation pathway, that being $tRNA^{Tyr(GUA)}$ [17, 18]. Recent work by Rubio *et al*. demonstrated that the intron of $tRNA^{Tyr(GUA)}$ is modified by editing events (base changes), and the editing events are required for efficient intron removal [17]. We compared the cytosine methylation pattern of unspliced versus spliced $tRNA^{Tyr(GUA)}$. The unspliced $tRNA^{Tyr(GUA)}$ has one cytosine in the intron. An analysis of this C indicates that <10% of the cytosines are resistant to bisulfite, suggesting that the intron is not a major target of cytosine methylation and that intronic cytosine methylation is not a prerequisite for splicing. However, both unspliced and spliced $tRNA^{Tyr(GUA)}$ exhibit robust methylation at the junction between the variable region and TψC stem. The levels of methylation are 84% for the unspliced $tRNA^{Tyr(GUA)}$ and 100% for the spliced $tRNA^{Tyr(GUA)}$. These data indicate that tRNA cytosine methylation in the exons can precede splicing, and the simplest model is that methylation typically precedes splicing as almost every unspliced $tRNA^{Tyr(GUA)}$ sequence displays cytosine methylation.

One potentially interesting finding in our studies is the absence of detectable methylation at C38. Three of the four *T. brucei* tRNAs that were analyzed contain a cytosine at position 38 and have the potential to be methylated; $tRNA^{Asp(GUC)}$, $tRNA^{Gly(GCC)}$, and $tRNA^{Val(AAC)}$. Although tRNAs from humans, mice, *D. melanogaster*, and *S. pombe* show evidence of C38 methylation in this position, it was not detectable in the three *T. brucei* tRNAs analyzed here. The lack of C38 methylation in *T. brucei* was not due to a technical issue, as we identified C38 methylation in 22/22 clones in $tRNA^{Asp(GUC)}$ analyzed from human placental RNA (data not shown). Thus, one simple model is that the cytosine 38 methylation pathway of *T. brucei* tRNAs is absent. Alternatively, it is possible that C38 tRNA methylation is present in another life cycle stage not examined in our studies, or under different growth conditions. We analyzed $tRNA^{Gly(GCC)}$ and $tRNA^{Val(AAC)}$ for cytosine methylation in bloodstream form parasites (single marker strain) as examples of tRNAs with high and low methylation levels in procyclic form parasites (Supplemental Figure 2). We did not observe methylation of C38 in either tRNA in bloodstream forms, although methylation at the variable region-TψC junction was present in both tRNAs, and relatively high levels of C40 methylation were detected for $tRNA^{Gly(GCC)}$. Yet, it is still possible that C38 methylation exists in other stages of the parasite's life cycle not examined in our study. The vast majority

of tRNAs do not harbor a cytosine at position 38, but it is not possible to rule out that *T. brucei* tRNAs other than those analyzed contain C38 methylation. *T. brucei* $tRNA^{His(GUG)}$, $tRNA^{Glu(CUC/UUC)}$, and $tRNA^{Leu(UAG)}$ all contain a predicted cytosine at position 38, but there is little evidence for C38 modification of these molecules in other organisms [12, 15]. In any case, further investigations will be required to determine if and when C38 is modified.

The lack of detectable C38 methylation may indicate that tRNA C38 methylation is an evolutionarily new modification compared to methylation at the junction between the variable region and TψC stem. C38 methylation is absent from bacteria and archaebacteria, but present in *S. pombe*, *Drosophila*, mice, and humans [5, 11-15]. It is important to note that *T. brucei* is an early branching eukaryote. One model for the evolution of tRNA methyltransferases is that bacterial DNA methyltransferases are ancestral to tRNA methyltransferases [7]. An analysis of the *T. brucei* genome indicates that there is one DNA methyltransferase homologue, TbDMT [8]. TbDMT contains the ten conserved motifs found in all enzymes that methylate DNA, and these ten motifs are also found in some enzymes that modify tRNA. Dnmt2 enzymes that modify tRNA have a conserved CFT motif adjacent to conserved motif IX. Interestingly, TbDMT is more homologous to bacterial DNA methyltransferases and the CFT motif is absent [8]. Thus, it is possible that TbDMT is not a member of the tRNA modification family, and this could explain the lack of detectable C38 methylation of *T. brucei* tRNAs. Several other protozoan parasites including *Plasmodium spp*., *Cryptosporidium spp*., and *Entamoeba histolytica* contain DNA methyltransferase homologs that do contain the CFT motif next to domain IX, and are homologous to Dnmt2 in contrast to bacterial DNA methyltransferases [7]. Thus, other protozoan parasites may have the ability to methylate C38 in tRNA, and the ability of the *E. histolytica* Dnmt2 homologue to methylate $tRNA^{Asp}$ *in vitro* is consistent with this model [19].

Three of the four *T. brucei* tRNAs analyzed here contain a cytosine at position 40 in the anticodon stem: $tRNA^{Gly(GCC)}$, $tRNA^{Asp(GUC)}$, and $tRNA^{Tyr(GUA)}$. Of the three tRNAs, only one showed evidence of methylation, that being $tRNA^{Gly(GCC)}$. Thus, *T. brucei* has the ability to methylate C40, yet not all tRNAs with a cytosine in this position are methylated. Furthermore, *T. brucei* $tRNA^{Gly(GCC)}$ displays partial methylation at C40, meaning that not all $tRNA^{Gly(GCC)}$ molecules are modified. Partial methylation of human $tRNA^{Gly(GCC)}$ has been observed [11], although the significance is unknown. Partial modification of $tRNA^{Gly(GCC)}$ suggests that there are two or more populations of $tRNA^{Gly(GCC)}$ with potentially different functions and/or the levels of modification at this site are controlled.

In many eukaryotic tRNAs, there are one to three methylated cytosines at the junction between the variable region and tRNA TψC stem [4]. Our sodium bisulfite sequencing data indicate that all *T. brucei* tRNAs analyzed have between one to three 5-methylcytosines in this region, and thus the pathway to generate cytosine methylation at the variable region-TψC junction is present in *T. brucei*. BLAST searches for the presence of *T. brucei* NSun2 homologues are positive, consistent with a model for active cytosine methylation in this region (data not shown). Interestingly, the levels of methylation at this site are different for different tRNAs. For $tRNA^{Gly(GCC)}$, all three Cs in the region are highly methylated. For $tRNA^{Asp(GUC)}$, two of the three cytosines are highly methylated, and one cytosine is

partially methylated (61%). For $tRNA^{Val(AAC)}$ and the unspliced and spliced $tRNA^{Tyr(GUA)}$ tRNAs, one of the two cytosines present is methylated. Thus, all four *T. brucei* tRNAs analyzed demonstrate cytosine methylation near the variable region-TψC junction, but the levels of methylation are different for different tRNAs. Thus, the presence of a cytosine at a particular methylation hotspot is not sufficient to dictate modification.

It is interesting to note that the number of modified cytosines in a *T. brucei* tRNA region does not always match eukaryotic counterparts. For example, *T. brucei* $tRNA^{Asp(GUC)}$ seems to be hypermethylated at the variable region-TψC junction as the *T. brucei* tRNA has three modified cytosines, but only one to two modified cytosines are found in this position in *S. pombe*, *Drosophila*, mouse and human $tRNA^{Asp(GUC)}$ [5, 11-14]. In contrast, *T. brucei* $tRNA^{Val(AAC)}$ has one 5-methylcytosine near the variable region-TψC junction, whereas mouse $tRNA^{Val(AAC)}$ has two 5-methylcytosines [11]. Thus, while the variable region-TψC junction hotspot is apparently present in numerous organisms, the level of cytosine methylation is organism dependent.

Our data demonstrating cytosine modification at the variable region-TψC junction are consistent with a model in which modification of this region is evolutionarily ancient. Although methylation at the variable region-TψC junction has not been identified in bacteria, it is present in some archaebacteria, the early branching eukaryote *T. brucei*, unicellular eukaryotes such as *S. pombe*, and metazoans [2, 5, 11-15]. The function of cytosine methylation at the variable region-TψC junction is starting to emerge, and the modifications may stabilize tRNA against degradation [11]. In *T. brucei*, the levels of tRNAs do not obviously correlate with gene copy number, and it is possible that differential stability of tRNAs via different numbers of methylated cytosines could contribute to different steady-state tRNA levels [20]. In humans, the enzyme that modifies cytosine near the variable region-TψC junction, NSun2, also generates 5-methylcytosine in mRNA [5], and thus is it is possible that other types of RNA in *T. brucei* contain modified bases including mRNA. Future experiments will focus on determining which types of RNA are modified in protozoan parasites, and elucidating functions for these modifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The *T. brucei* tRNAs analyzed contain between 1-4 5-methylcytosines.
- Cytosine residues near the variable region-TψC junction are frequently methylated.
- *T. brucei* $tRNA^{Gly(GCC)}$ displays cytosine methylation at C40 of the anticodon stem.
- There is no evidence for 5-methylcytosine at C38 in the *T. brucei* anticodon loop.

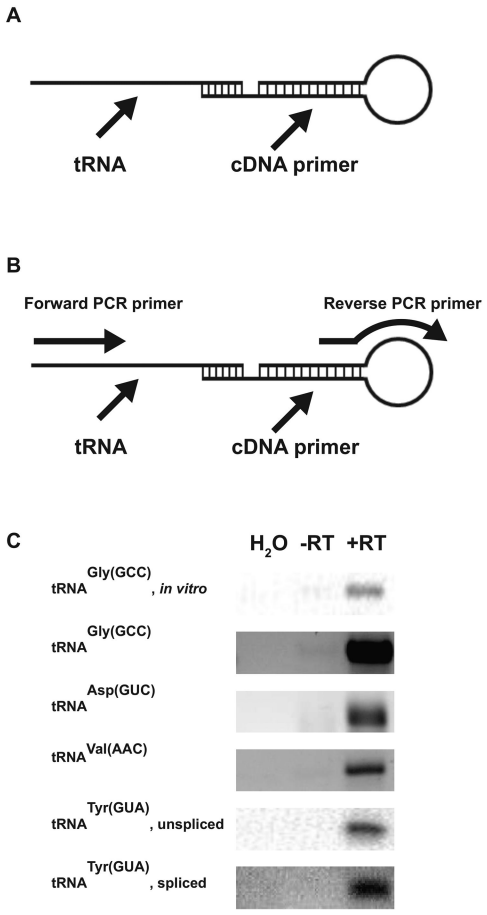

Figure 1. PCR amplification of sodium bisulfite treated small RNAs.

Small RNAs were treated with sodium bisulfite and used as a template for cDNA synthesis. A) Primers for cDNA synthesis hybridize to the 3′ end of the tRNA and contain a stem loop. Each primer hybridizes to 7-12 nucleotides at the 3′ end of the tRNA. B) The forward PCR primer binds to the 5′ end of the tRNA. The reverse PCR primer binds to the stem loop. The cDNA primers and forward PCR primers were designed to be complementary to the deaminated cDNA assuming no modified cytosines in the priming region. C) PCRs either lacked template (H_2O), contained mock cDNA made in the absence of reverse transcriptase (-RT), or contained cDNA made in the presence of reverse transcriptase (+RT). Reactions were separated by agarose gel electrophoresis and stained with GelRed.

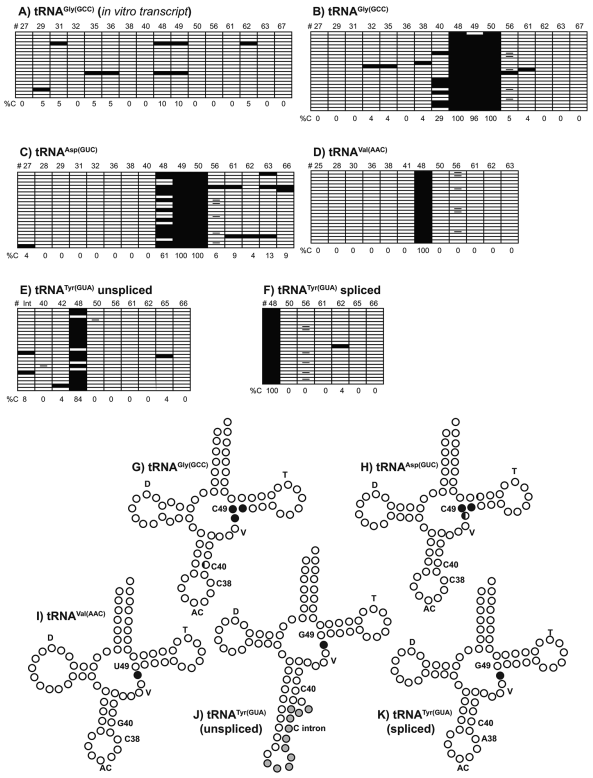

Figure 2. Sodium bisulfite sequencing analysis of procyclic form *T. brucei* tRNAs.

PCR products from bisulfite treated small RNAs were analyzed by DNA sequencing. A-F) Diagrams of modification patterns of *in vitro* synthesized $tRNA^{Gly(GCC)}$ A) and native *T. brucei* tRNAs (B-F). Each horizontal row represents one sequencing reaction. Open boxes represent Ts, filled boxes represent Cs, and minus signs (-) represent missing sequencing information in individual clones. Numbers on the top of each diagram represent the position of individual Cs in the tRNA (#), and numbers at the bottom of each diagram represent the percent of sequences with Cs in a particular position (%C). G-K) Patterns of modification with respect to predicted tRNA secondary structures. D is the D-loop, T is the TψC loop, V is the variable region, and AC is the anticodon loop. For unspliced $tRNA^{Tyr(GUA)}$ (J), gray circles represent bases in the intron. Positions 38, 40, and 49 are labeled for reference. Open circles, cytosines with 10% or less modification (or non-cytosines); ¼ filled circles, cytosines with 11-40% modification; ½ filled circles, cytosines with 41-70% modification; and completely filled circles, cytosines with 71-100% modification.

Table I

Oligonucleotides used for reverse transcription and amplification of bisulfite treated small RNA

Target	Exp[c]	Oligonucleotides(s)[d]
$tRNA^{Asp(GUC)}$	RT	5′CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGGCTTCCCA3′
$tRNA^{Asp(GUC)}$	PCR	5′TTTTTGGTAGTATAGTGG3′, 5′CTGGTGTCGTGGAGT3′
$tRNA^{Gly(GCC)}$	RT	5′CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGGTAATACACA3′
$tRNA^{Gly(GCC)}$	PCR	5′GTAGTTGGTTTAGTGGTAGA3′, 5′CTGGTGTCGTGGAGT3′
$tRNA^{Tyr(GUA)}$, U[a]	RT	5′CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGGTCCTTCC3′
$tRNA^{Tyr(GUA)}$, U[a]	PCR	5′AATTGGTAGAGTATGTGATTGTAGAGTATG3′, 5′CTGGTGTCGTGGAGT3′
$tRNA^{Tyr(GUA)}$, S[b]	RT	5′CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGGTCCTTCC3′
$tRNA^{Tyr(GUA)}$, S[b]	PCR	5′GAGTATGTGATTGTAGATTATAGGGT3′, 5′CTGGTGTCGTGGAGT3′
$tRNA^{Val(AAC)}$	RT	5′GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGTAATACACCAAT3′
$tRNA^{Val(AAC)}$	PCR	5′TGATGGTTTAGGTGGTTATGA3′, 5′GTGCAGGGTCCGAGGT3′

[a] U, unspliced

[b] S, spliced

[c] RT, reverse transcription

[d] stem loops in RT primers are underlined