METHOD

Sensitive and quantitative probing of pseudouridine modification in mRNA and long noncoding RNA

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ABSTRACT

Pseudouridine (Ψ) is the most abundant RNA modification in cellular RNA present in tRNA/rRNA/snRNA and also in mRNA and long noncoding RNA (lncRNA). Elucidation of Ψ function in mRNA/lncRNA requires mapping and quantitative assessment of its modification fraction at single-base resolution. The most widely used Ψ mapping method for mRNA/lncRNA relies on its reaction with *N*-Cyclohexyl-*N'*-(2-morpholinoethyl)carbodiimide (CMC), forming an adduct with the Ψ base in RNA that is detectable by reverse transcription (RT) stops. However, this method has not produced consistent Ψ maps in mRNAs; furthermore, available protocols do not lend confidence to the estimation of Ψ fraction at specific sites, which is a crucial parameter for investigating the biological relevance of mRNA modifications. Here we develop a quantitative RT-PCR based method that can detect and quantify the modification fraction of target Ψ sites in mRNA/lncRNA, termed CMC-RT and ligation assisted PCR analysis of Ψ modification (CLAP). The method still relies on RT stop at a CMC- Ψ site, but uses site-specific ligation and PCR to generate two distinct PCR products in the same sample, corresponding to the modified and unmodified site, that are visualized by gel electrophoresis. CLAP not only requires a small amount of cellular RNA to validate Ψ sites but also determines the Ψ fraction semiquantitatively at target sites in mRNA/lncRNA. We determined the Ψ status of four mRNA sites and one lncRNA site whose modification fractions range from 30% to 84% in three human cell lines. Our method enables precise mapping and assessment of Ψ modification levels in low abundance cellular RNAs.

Keywords: pseudouridine; mRNA; RT-PCR

INTRODUCTION

More than 170 types of RNA modifications have been identified in biology (Delaunay and Frye 2019). Pseudouridine (Ψ) was the first RNA modification discovered and is also the most abundant in cellular RNA (Charette and Gray 2000). Ψ is the 5-ribosyl isomer of uridine (U) with a carbon-carbon (C5-C1') bond in place of the nitrogen-carbon (N1-C1') bond between the base and the sugar backbone. The C-C bond gives Ψ more rotational freedom and conformational flexibility (Charette and Gray 2000; Ge and Yu 2013). In addition, Ψ has an extra hydrogen bond donor at the N1 position. Ψ modification has been shown to rigidify RNA structure through hydrogen bonding to the ribose-phosphate backbone and enhance RNA stacking. Ψ in rRNA and tRNA has been shown to fine-tune and stabilize the regional structure and help maintain their functions in mRNA decoding, ribosome assembly, processing, and translation (Charette and Gray 2000; Ge and Yu 2013; Li et al. 2016; Rintala-Dempsey and Kothe 2017). Ψ in

snRNA has been shown to enhance spliceosomal RNA-pre-mRNA interaction to facilitate splicing regulation (Wu et al. 2011, 2016). A recent study unveiled a role for pseudouridylated tRNA fragments in translational control of stem cells (Guzzi et al. 2018).

Ψ has also been found in mRNA and long noncoding RNA (lncRNA) (Carlile et al. 2014; Lovejoy et al. 2014; Schwartz et al. 2014; Li et al. 2015). Ψ is among the major mRNA modifications identified; its overall abundance as measured by mass spectrometry (Li et al. 2015) is second only to *N*⁶-methyladenosine (*m*⁶A). Additional Ψ modifications appear in the transcriptome under stress (Carlile et al. 2014; Schwartz et al. 2014), indicating that it is dynamic and plays multiple roles in the regulation of gene expression in a cell type- and cell state-dependent manner. A well-defined function of Ψ in mRNA is to enable efficient stop codon readthrough (Karjane and Yu 2011).

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To elucidate the biological function of Ψ in mRNA/lncRNA, it is crucial to validate the location of Ψ sites in low abundance RNA species. Like all mRNA/lncRNA modifications studied so far, the Ψ modification fraction at each mRNA site may also be highly variable and likely plays a role in regulating its function. At this time, all high-throughput sequencing methods rely on the reaction of *N*-cyclohexyl-*N'*-(2-morpholinoethyl)carbodiimide (CMC) with Ψ . CMC forms an adduct with Ψ residues in RNA after a series of chemical treatments. The CMC- Ψ adduct can be detected by primer extension methods that result in a reverse transcriptase (RT) stop (Bakin and Ofengand 1993) and/or occasional RT readthrough that leaves behind a mutation signature in the sequencing reads (Lei and Yi 2017; Zhou et al. 2018). The RT-stop method is the most commonly used approach. In either case, the multiple chemical handling steps generate higher than desired background in the sequencing reaction, so that mapping at the mRNA/lncRNA transcriptome level has not been consistent among different studies (Li et al. 2016; Safra et al. 2017). Furthermore, transcriptome-wide mapping methods have not generated reliable information on the Ψ modification fraction at individual sites which is a biological variable in the response to cellular conditions.

Although candidate Ψ sites in abundant rRNA or snRNA can be validated by primer extension and denaturing gel analysis or thin-layer chromatography (Zhao and Yu 2004), validation of Ψ sites in low abundance mRNA/lncRNA has been challenging. In principle, a SCARLET-based method can be used to validate and quantify a target Ψ site in an mRNA (Liu et al. 2013), but this method requires radioactivity and requires a very large amount of input sample. Yi and colleagues developed a qPCR-based method for locus-specific detection of Ψ (Lei and Yi 2017). This method relies on induced mutation/deletion in cDNA synthesis by the Superscript II RT; the resulting mutation/deletion PCR products generate distinct melting curves after RT-PCR. Although this method is radiolabel free and useful in identifying Ψ modification in mRNA, it cannot readily obtain quantitative information of Ψ fraction at individual sites.

Here we develop an RT-PCR- and gel electrophoresis-based method that validates and quantifies Ψ sites in mRNA/lncRNA at single-base resolution. Our method, termed CMC-RT and Ligation Assisted PCR analysis of Ψ modification (CLAP), still relies on the initial generation of the CMC- Ψ adduct at a target site and the RT stop it induces, but the RT stop product is selectively ligated to an oligonucleotide that subsequent PCR generates two different-sized products corresponding to the Ψ modified and unmodified target RNA in the same sample using a single set of PCR primers. This uniform amplification of cDNAs derived from Ψ modified and unmodified RNA produces quantitative information for individual Ψ sites. We first show that CLAP is quantitative in determining Ψ

modification using an RNA standard and applying it to several rRNA Ψ sites. We then apply CLAP to four target mRNAs and one target lncRNA Ψ site previously identified by high-throughput sequencing. We validate the presence of Ψ modification and obtain quantitative information about these mRNA/lncRNA sites in three human cell lines, thus demonstrating the utility of the method for quantitative investigations.

RESULTS AND DISCUSSION

Ψ in an RNA standard and rRNA

The unique feature of the CMC reaction with U-like and G-like residues has been known since the 1970s and was first applied to identify Ψ in rRNA and tRNA in 1993 by primer extension (Ho and Gilham 1971; Bakin and Ofengand 1993). CMC can react with U, G, and Ψ residues to form N3-CMC and N1-CMC adducts. All except the N3-CMC- Ψ adducts are readily removed under alkaline conditions. In primer extension, the bulky N3-CMC group of Ψ stops the reverse transcriptase one nucleotide 3' to the Ψ residue. This classical method has been adapted to map Ψ sites in the transcriptome at hundreds or even thousands of sites (Carlile et al. 2014; Lovejoy et al. 2014; Schwartz et al. 2014; Li et al. 2015). The difficulty in using this method for low abundance mRNA transcripts is that sequencing reads that appear to be RT stops in the CMC-treated samples can be derived from multiple sources, which increases the noise for calling a Ψ site. Thus, quantitative assessment of Ψ fraction has not been reliable.

We developed a scheme to apply the CMC-based method for Ψ analysis, but with the goal of validating Ψ identification and at the same time quantifying the Ψ modification level at a target site (Fig. 1A). We reasoned that the RT stop product derived from a target CMC-treated Ψ site can be selectively amplified by ligating a DNA oligo guided by a complementary DNA splint to introduce the second PCR primer binding site. A crucial feature of our method is to make the second PCR primer binding site identical to a downstream region in the contiguous cDNA derived from the unmodified transcript encompassing the upstream RNA region to the target Ψ site so that a single set of PCR primers can be used to amplify both the RT-stopped and contiguous cDNA products simultaneously. Our design allows for the PCR products to have different lengths, a short one corresponding to the Ψ modified transcript and a ~30 bp longer product corresponding to the unmodified transcript. The application of a single set of PCR primers provides the uniformity needed to quantify both short and long PCR products in the same reaction and in the same lane of a native gel. In contrast, conventional PCR methods involve two primer sets for RT-stop quantitation and use a subtractive strategy from two different PCR reactions, which increases a

FIGURE 1. Schematics of the CLAP method and testing with U/Ψ-containing RNA standards. (A) Schematics. RNA-5 is a 5mer RNA oligo used to block the randomly cleaved RNA during the CMC treatment. RNA is shown as an orange line, RNA-5 as black line, cDNA as a green line, and the ligated primer binding site in Ψ + CMC as the red line at the end, with the same primer binding site in contiguous cDNA indicated as an embedded red line. PCR primers are represented by red and blue arrows. (B) Native PAGE showing two PCR bands derived from the U- and Ψ-containing RNA standard after the CLAP procedure. All samples contained 30 fmole of 100mer RNA standard at indicated Ψ fractions spiked into 1 μg HEK293T total RNA. (C) Quantification of the result from panel B. The measured Ψ fraction is calculated by subtracting the fraction of the short product band in the -CMC lane from that of the +CMC lane. (D) Native PAGE showing the CLAP result of a 1:1 U/Ψ RNA standard mixture at different spike-in amounts added to 1 μg HEK293T total RNA. (E) Quantification of the result from panel D.

nonuniform bias in the PCR reaction (Vandenbroucke et al. 2001). Although multiplex PCR can also be used to detect different cDNA products, the need to use different PCR primer pairs may also impose a PCR amplification bias, and the ratio of the PCR products cannot be visualized in a single lane (Singh et al. 2000). We termed our method CLAP which combines RT stops by CMC-Ψ, splint ligation, single-set primer PCR, and single lane visualization.

We first used a 100mer RNA standard to demonstrate the feasibility and the quantitative nature of the method (Fig. 1B). We mixed the fully Ψ-modified or unmodified 100mers at defined ratios and spiked the mixture into the HEK293T total RNA to mimic the subsequent analysis of mRNA transcripts. We started with an optimized CMC reaction with Ψ (Supplemental Fig. S1; Zhou et al. 2018) to obtain a nearly stoichiometric level of CMC-Ψ adduct. CMC procedures rely on harsh conditions and produce a higher than desired level of RNA fragmentation.

The specific RNA cleavage product 3' to the target Ψ site would have produced the same RT product, thus increasing the background noise. To reduce this background, we first ligated a 5mer blocking RNA oligo to the free 5' end of RNA cleavage products after the CMC treatment before carrying out the RT reaction (the RNA-5 ligation step in Fig. 1A). By optimizing the ligation conditions, we were able to reduce the non-Ψ derived, short PCR product by at least fourfold (Supplemental Fig. S2). We performed RT reactions using the avian myoblastoma virus (AMV) reverse transcriptase, which is known to stop most frequently at Ψ-CMC adducts. In the CMC-treated sample, RT reaction would generate two cDNA products: a short one from the RT stop one nucleotide 3' to the Ψ site and a long product from contiguous cDNA synthesis of the unmodified RNA. Guided by a complementary DNA oligo splint, we ligated the RT-stop cDNA product to a DNA oligonucleotide containing the identical forward

PCR primer binding site >30 bp away from the Ψ site. Finally, we carried out PCR using just one set of forward and reverse primers to generate two PCR products corresponding to the Ψ -modified and unmodified RNA transcript. Analysis of the PCR products by native gel electrophoresis enabled the determination of Ψ modification fraction. Using ~30 fmole of the 100mer RNA standard (1 ng) spiked into one microgram of total RNA, we found that the CLAP method can quantitatively assess the input Ψ fraction (Fig. 1B,C). We also estimate from the calibration curve (Fig. 1C) that a Ψ site modified at ~5% level should be detectable using a twofold threshold above the background. To test the sensitivity of the CLAP method, we added a 1:1 mixture of Ψ /U-RNA standard to HEK293T total RNA at different concentrations. CLAP was able to precisely quantify the Ψ fraction at a ~1.9 fmol level in one microgram of total RNA (~60 pg/ μ g; Fig. 1D,E). Using an estimate of total poly(A)⁺ RNA constituting ~3% of total RNA, this level corresponds to ~0.2% of total mRNA. In principle, CLAP should be able to analyze mRNA transcripts at even lower abundance upon increasing the PCR cycle number and additional optimization of PCR conditions.

To further validate the CLAP method, we examined three rRNA Ψ sites that can be independently tested by visualizing the CMC- Ψ derived RT stop products without amplification (Fig. 2). We chose one Ψ site (Ψ 822) in 18S rRNA and two Ψ sites (Ψ 3749 and Ψ 4412) in 28S rRNA (Piekna-Przybylska et al. 2008; Taoka et al. 2018). Applying primer extension after CMC treatment, we found

the Ψ levels to be 84% for 18S Ψ 822, 74% for 28S Ψ 3749, and 66% for 28S Ψ 4412 (Fig. 2A). Applying the CLAP method, we determined the Ψ levels to be 83% for 18S Ψ 822, 74% for 28S Ψ 3749, and 71% for 28S Ψ 4412 (Fig. 2B). This agreement between the primer extension and CLAP methods supports the feasibility of using CLAP for quantitative analysis of Ψ modifications. The modification fraction of the same three sites detected by mass spectrometry using TK6 rRNA were all >99% (Taoka et al. 2018). This result suggests that the CLAP method is semiquantitative to measure the absolute Ψ modification fraction and may underestimate the Ψ fraction by up to 1.4-fold, likely due to either incomplete CMC- Ψ adduct formation and/or a small amount of CMC- Ψ reversal in the CMC procedure.

Ψ in mRNA and lncRNA

Next, we examined the Ψ status of two Ψ sites in the abundant eEF1A1 mRNA and one Ψ site in MALAT1 lncRNA. eEF1A1 is an isoform of the α subunit of the elongation factor-1 complex and plays an essential role in translation by delivering aminoacyl-tRNA to the ribosome. This mRNA has been frequently used as a reference for qPCR studies (Gentile et al. 2016; Mughal et al. 2018). MALAT1 is a metastasis-promoting lncRNA and plays a role in alternative splicing, nuclear organization, epigenetic modulation of gene expression, synapse formation, and myogenesis (Zhang et al. 2017). Previous Ψ sequencing (CeU-seq, Li et al. 2015) identified two Ψ sites in eEF1A1 (Ψ 519 and Ψ 875) and one Ψ site in MALAT1 (Ψ 5590). These sites have been validated by a qPCR-based Ψ detection method (Lei and Yi 2017). Applying the CLAP method, we determined the modification level of eEF1A1 Ψ 519 at $31 \pm 2\%$, eEF1A1 Ψ 875 at $56 \pm 3\%$, and MALAT1 Ψ 5590 at $84 \pm 5\%$ in HEK293T cells (Fig. 3A). The nearly complete pseudouridylation of the MALAT1 site is consistent with the high modification level (~75%) estimated by a qPCR-based method (Lei and Yi 2017). We also examined these Ψ sites in HeLa and MCF7 cell lines (Fig. 3B). eEF1A1 Ψ 875 and MALAT1 Ψ 5590 had similar Ψ modification levels in all three cell lines; in contrast, eEF1A1 Ψ 875 levels ranged from $56 \pm 3\%$ in HEK293T to $35 \pm 2\%$ in MCF7.

To investigate the sensitivity of the CLAP method we chose two Ψ sites in low abundance mRNAs, hypoxanthine phosphoribosyltransferase 1 (HPRT1), and proteasome activator subunit 2 (PSME2) identified by CeU-seq (Li et al. 2015; Lei and Yi 2017). Both mRNAs are present at <1% the abundance of the eEF1A1 mRNA according to mRNA-seq analysis in HEK293T cells (Liu et al. 2017). We used 1 μ g HEK293T total RNA to successfully measure the Ψ status of HPRT1 Ψ 733 and PSME2 Ψ 616. Applying the CLAP method, we determined that the modification level of HPRT1 Ψ 733 was $51 \pm 1\%$ and of PSME2 Ψ 616 $62 \pm 5\%$ (Fig. 4A). To validate the accuracy and sensitivity

FIGURE 2. Quantification of Ψ levels in rRNA. (A) Primer extension of the 18S Ψ 822, 28S Ψ 3749, and 28S Ψ 4412 sites. The RT stops are marked by a triangle. The estimated Ψ modification levels are 74% for Ψ 3749, 84% for Ψ 822, and 66% for Ψ 4412. (B) CLAP result for the same three rRNA Ψ sites. The estimated Ψ modification levels are $74 \pm 3\%$ for Ψ 3749, $83 \pm 1\%$ for Ψ 822, and $71 \pm 1\%$ for Ψ 4412.

FIGURE 3. CLAP analysis of Ψ in high abundance mRNA and lncRNA transcripts. (A) CLAP examination of eEF1A1 Ψ519, eEF1A1 Ψ875, and MALAT1 Ψ5590 sites using 1 μg HEK293T total RNA. (B) Ψ modification levels in three cell lines, three biological replicates for each site.

of measuring Ψ status in low abundance mRNA in the context of total RNA by CLAP, we also used 50 ng poly(A)-selected RNA as input for the CLAP procedure (Fig. 4B). We found similar levels for HPRT1 Ψ733 [51% in total RNA and 46% in poly(A)-selected RNA] and a 1.4-fold higher level for PSME2 Ψ616 [62% in total RNA and 86% in poly(A)-selected RNA]. Both sites showed similar modification levels among the three cell lines examined (Fig. 4C).

To assess the functional relevance of these 5 Ψ sites, we performed phylogenetic analysis among the vertebrate lineage (Supplemental Fig. S3) using Jalview (Waterhouse et al. 2009). The four mRNA Ψ-containing regions are conserved across vertebrates, whereas the Ψ5590-containing region is only present in the MALAT1 RNA from primates. All four mRNA sites are in the coding regions and part of a Val codon. The eEF1A1 Ψ519 site is in the third codon position (GUΨ); this uridine is sometimes changed to C, which still maintains the amino acid identity of the codon. eEF1A1 Ψ875, HPRT1 Ψ733, and PSME2 Ψ616 sites are in the second codon position (GΨU, GΨA); these uridines are unchanged in all vertebrates. The conserved Ψ

modification in the second codon position may be useful for decoding these Val codons.

In summary, we developed a sensitive and quantitative RT-PCR method for site-specific analysis of Ψ modification. Our method can validate candidate Ψ sites in target mRNA and lncRNA using total RNA and directly visualize the modification levels. Since CLAP makes use of RT stops, in principle it can be applied to site-specific detection and quantitation of other RNA modifications that induce RT stops during cDNA synthesis. These include the Watson–Crick face RNA methylations of *N*¹-methyladenosine (*m*¹A), *N*³-methylcytidine (*m*³C), *N*¹-methylguanosine (*m*¹G), *N*², *N*²-dimethylguanosine (*m*²2G), and *N*³-methyluridine (*m*³U). *m*¹A has already been shown to be present in mammalian mRNAs, but its modification levels at target sites have not been investigated (Dominissini et al. 2016; Li et al. 2017). A potential limitation of the CLAP method is for closely spaced two or more Ψ sites in the same RNA, it can only accurately assess the 3' most Ψ site.

Among the mRNA/lncRNA target sites we analyze here, Ψ level ranges from 30% to 85%, which is compatible to the more abundant *m*⁶A modification in mRNA/lncRNA. Quantitative measurement of the modification level is an important biological feature in the epitranscriptome that has not received much attention. The quantitative nature of our CLAP method in the determination of Ψ status in low abundance mRNAs should enable the investigation of cell type- and cell state-dependent variations in Ψ levels.

MATERIALS AND METHODS

Cell culture and RNA extraction

HEK293T, HeLa, and MCF7 cells were cultured under standard conditions. Briefly, HEK293T and HeLa cells were grown in Hyclone DMEM medium (GE Healthcare Life Sciences, SH30022.01) with 10% FBS and 1% Pen–Strep (Penicillin–Streptomycin) to 80% confluency. MCF7 cells were grown in EMEM medium (ATCC, 30-2003) with 10% FBS, 1% Pen–Strep, 0.01 mg/mL bovine insulin (Sigma-Aldrich, I0516), and 10 nM β-estradiol (Sigma-Aldrich, E2758) to 80% confluency. Total RNA was extracted using TRIzol reagent (ThermoFisher, 15596026) following the manufacturer's protocol. Poly(A)⁺ RNA was enriched using the PolyATract mRNA Isolation System (Promega, Z5310) following the manufacturer's instructions.

In vitro transcription of U- or Ψ-containing control oligos

The 100-nt U- or Ψ-containing RNA standard was in vitro transcribed using T7

FIGURE 4. CLAP analysis of Ψ in low abundance mRNA transcripts. (A) CLAP results for HPRT1 Ψ733 and PSME2 Ψ616 using 1 μg of HEK293T total RNA as input. (B) CLAP quantitation of HPRT1 Ψ733 and PSME2 Ψ616 using HEK293T total RNA (1 μg input) or poly(A)⁺ RNA (50 ng input). (C) Ψ modification levels in three cell lines, three biological replicates for each site.

RNA polymerase. In vitro transcription took place in 1× RNA polymerase buffer; 14 mM MgCl₂; 5 mM DTT; 4 mM each ATP, GTP, CTP; 4 mM UTP or ΨTP (TriLink BioTechnologies, N-1019); 10 U/μL T7 RNA polymerase (NEB, M0251L); and 85 nM dsDNA template. The whole mixture was incubated at 37°C for 2.5 h. Then 1/10th volume of 3 M NaOAc/HOAc (pH 5.2) and 3× volume of 100% ethanol were added to the mixture to precipitate the RNA oligos. The precipitated RNA oligos were purified using 10% denaturing gels containing 7 M urea.

The sequence of the RNA standard is as follows:

5'-GGGAGGCGAGAACACACCACAACGAAAACGAGCAAAA
CCCGG(Ψ/U)ACGCAACACAAAAGCGAACAACGCGAAAAA
GGACACCGAAGCGGAAGCAAAGACAAC-3'

CMC treatment of RNA

Total RNA or poly(A)⁺ RNA was CMC [*N*-cyclohexyl-*N'*-(2-morpholinoethyl)carbodiimide] labeled as previously described (Zhou et al., 2018). A major change in this work is the increased CMC reaction time (~16 h) at lower temperature (30°C) to ensure nearly complete conversion of Ψ to Ψ-CMC while minimizing RNA degradation. Briefly, 10 μg total RNA or poly(A)⁺ RNA in 12 μL water was mixed with 24 μL of 1× TEU buffer consisting of 50 mM Tris-HCl (pH 8.3), 4 mM EDTA, 7 M urea, and 4 μL of 1 M freshly prepared CMC in TEU buffer (+CMC) or just 4 μL TEU buffer (–CMC) for a final condition of 0.7× TEU buffer in 40 μL. The CMC reaction was carried out at 30°C for 16 h. To remove excess CMC, the following reagents were added to the reaction mixture: 160 μL of 50 mM KOAc (pH 7), 200 mM KCl; 3 μL of 5 μg/μL glycogen; and then 550 μL of ethanol. The mixture was incubated at –80°C for >2 h, then centrifuged at 15 krpm in a micro-centrifuge for 30 min. After removing the supernatant, the pellet was mixed with 500 μL of 75% ethanol and then kept at –80°C for at least 2 h and centrifuged once more. The washing and precipitation steps were repeated one more time. To reverse the CMC-U/CMC-G adducts, the RNA pellet was mixed with 40 μL of 50 mM Na₂CO₃, 2 mM EDTA (pH 10.4), and incubated at 37°C for 6 h. The reaction mixture was then mixed with 160 μL of 50 mM KOAc (pH 7), 200 mM KCl, followed by ethanol precipitation. The RNA recovery yield for this protocol was >65%.

RNA 5' phosphorylation and RNA-5 blocking oligo ligation

To reduce the signal derived from RNA fragmentation during the CMC reaction, ±CMC-treated RNA in 6.5 μL H₂O was mixed with 0.5 μL RNase inhibitor (NEB, M0307L), 1 μL of 10× T4 PNK reaction buffer A, 1 μL of 1 mM ATP, 1 μL of T4 Polynucleotide kinase (PNK), and then incubated at 37°C for 30 min. To ligate the RNA-5 oligo (/5AmMC6/rArCrCrCrA; Integrated DNA Technologies), 1 μL of 10× T4 RNA Ligase Reaction Buffer, 1 μL of 100 μM RNA-5 oligo, 1 μL of 1 mM ATP, 1 μL of RNase inhibitor, 3 μL of DMSO, 2 μL of H₂O, and 1 μL of T4 RNA ligase I (NEB, M0437M) were added to this mixture and incubated at 16°C for 16 h. The reaction was terminated upon the addition of 1.2 μL of 200 mM EDTA.

Reverse transcription and splint ligation

Reverse transcription was carried out using 3 μL of the above ligation mixture (~1 μg RNA) using AMV reverse transcriptase (NEB,

M0277L) and target-specific primers. To anneal the primer, 1 μL of 10× annealing buffer (250 mM Tris-HCl [pH 7.4], 480 mM KCl) and 1 μL of 0.5 μM target-specific RT primer were added, and the mixture was incubated at 93°C for 2 min. To start the RT reaction, 5 μL of 2× AMV RT reaction mixture was added for a final condition of 0.6 U/μL AMV RT, 1× AMV RT buffer, and 0.5 mM of each dNTP. The RT reaction proceeded at 42°C for 1 h. After incubation at 85°C for 5 min to inactivate RT, 1 μL of 5 U/μL RNase H (NEB, M0297L) was added and the mixture incubated at 37°C for 20 min to digest the RNA. RNase H was inactivated by heating the mixture at 85°C for 5 min.

To anneal the adaptor, 1 μL of the adaptor/splint oligos mixture (1.5 μM each) was added to the above RT mixture, followed by incubation at 75°C for 3 min. To ligate the adaptor, 4 μL of 4× ligation mixture was added for a final concentration of 10 U/μL of T4 DNA ligase (NEB, M0202L), 1× T4 DNA ligase reaction buffer, and 12.5% DMSO. The ligation proceeded at 16°C for 16 h. DNA ligase was inactivated by heating the mixture at 65°C for 10 min.

PCR amplification and gel electrophoresis

To perform PCR, 2 μL of the above ligation mixture was mixed with various components for a final condition of 1× Q5 reaction buffer, 1× Q5 high GC enhancer, 200 μM of each dNTP, 0.5 μM forward and reverse primers, and 0.02 U/μL Q5 high-fidelity DNA polymerase (NEB, M0491L); the final PCR reaction volume was 35 μL. PCR was done for high abundance eEF1A1 mRNA and MALAT1 RNA sites at 15 cycles and for low abundance PSME2 and HPRT1 sites 25–35 cycles.

Half of the PCR mixture (17.5 μL) was mixed with 3.5 μL of 6× TriTrack DNA Loading Dye (ThermoFisher, R1161). The entire mixture was loaded on a prerun 10% nondenaturing gel containing 1× TBE, together with low range DNA ladder (ThermoFisher, SM1193). The gel was stained with SYBR gold nucleic acid gel stain (ThermoFisher, S11494) for 10 min. Product bands were visualized using the Bio-Rad ChemiDoc imaging system and the bands quantified using Image Lab.

CLAP primer and oligonucleotide sequences

PseudoU_Ctrl RT Primer: 5'-GTTGTCTTTGCTTCCGCTTCG-3'

PseudoU_Ctrl Adaptor: 5'-pCCATGGGTGTGTTCTCGCCTCCC-3'

PseudoU_Ctrl Splint: 5'-ACACCCATGGACGCAACACA/3SpC3/-3'

PseudoU_Ctrl Forward PCR Primer: 5'-GGGAGGCGA GAACACACC-3'

28S Ψ3749 RT Primer: 5'-TCGTTTCATCCATTCATGCGC-3'

28S Ψ3749 Adaptor: 5'-pCCATGGCGCTTCATTGAATTCTTCAC-3'

28S Ψ3749 Splint: 5'-AGCGCCATGGCTTAAGGTAG/3SpC3/-3'

28S Ψ3749 Forward PCR Primer: 5'-GTGAAGAAATTCAA TGAAGCGC-3'

18S Ψ822 RT Primer: 5'-AACCGCGGTCCTATTCCATTA TT-3'

18S Ψ822 Adaptor: 5'-pCCATGGACACTCAGCTAAGA GCATCG-3'

18S Ψ822 Splint: 5'-GTGTCCATGGTCAAAGCAGG/ 3SpC3/-3'

18S Ψ822 Forward PCR Primer: 5'- CGATGCTCTTA GCTGAGTGTC-3'

28S Ψ4412 RT Primer: 5'-TGCTTCACAATGATAGGAAGA GCC-3'

28S Ψ4412 Adaptor: 5'-pCCATGCCGCCACAAGCCA GTTAT-3'

28S Ψ4412 Splint: 5'-GGCGGCATGGCCTTCGATGT/ 3SpC3/-3'

28S Ψ4412 Forward PCR Primer: 5'-ATAACTGGCTTG TGGCGG-3'

EEF1A1 Ψ519 RT Primer: 5'-ATATCTCTTCTGGCTG TAGGGTG-3'

EEF1A1 Ψ519 Adaptor: 5'-pCCATGGCCAGAAGGGC ATGCTCT-3'

EEF1A1 Ψ519 Splint: 5'-CTGGCCATGGAACAAAATGG/ 3SpC3/-3'

EEF1A1 Ψ519 Forward PCR Primer: 5'-AGAGCATG CCCTTCTGGC-3'

EEF1A1 Ψ875 RT Primer: 5'-TGCATTTGACAGATTTTAC TTCCG-3'

EEF1A1 Ψ875 Adaptor: 5'-pCCATGTACCACCAATTT GTAGACATCCTG-3'

EEF1A1 Ψ875 Splint: 5'-TGGTACATGGTCTCAAACCC/ 3SpC3/-3'

EEF1A1 Ψ875 Forward PCR Primer: 5'-CAGGATGTCTA CAAAATTGGTGGTA-3'

MALAT1 Ψ5590 RT Primer: 5'-TAAAGATGCAAATG CCTCTGAGTGA-3'

MALAT1 Ψ5590 Adaptor: 5'-pCCATGAGGAGAAAGTG CCATGGTTGATATT-3'

MALAT1 Ψ5590 Splint: 5'-CTCCTCATGGAGGACTTGTT/ 3SpC3/-3'

MALAT1 Ψ5590 Forward PCR Primer: 5'-AATATCAACCA TGGCACTTTCTCCT-3'

HPRT1 Ψ733 RT Primer: 5'-GACACAAACATGATTCAAAT CCCTG-3'

HPRT1 Ψ733 Adaptor: 5'-pCCATGAAAGTCTGGCTTATA TCCAACACTT-3'

HPRT1 Ψ733 Splint: 5'-ACTTTCATGGAGGATATGCC/ 3SpC3/-3'

HPRT1 Ψ733 Forward PCR Primer: 5'-AAGTGTGGATA TAAGCCAGACTTT-3'

PSME2 Ψ616 RT Primer: 5'-TATGATAAAGCTCAGCATAG AAGGC-3'

PSME2 Ψ616 Adaptor: 5'-pCCATGCGTTCTGAGAAG TACTTGGAATGG -3'

PSME2 Ψ616 Splint: 5'-GAACGCATGGAATGGATTAC/ 3SpC3/-3'

PSME2 Ψ616 Forward PCR Primer: 5'-CCATTTCCAAGTA CTTCTCAGAACG-3'

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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