Direct and site-specific quantification of RNA 2′-O-methylation by PCR with an engineered DNA polymerase

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ABSTRACT

Methylation of the 2′-hydroxyl-group of ribonucleotides is found in all major classes of RNA in eukaryotes and is one of the most abundant posttranscriptional modifications of stable RNAs. In spite of intense studies, the multiple functions of RNA 2′-O-methylation are still not understood. One major obstacle in the field is the technical demanding detection methods, which are typically laborious and do not always deliver unambiguous results. We present a thermostable KlenTaq DNA polymerase variant with significant reverse transcription activity that is able to discriminate 2′-O-methylated from unmethylated RNAs. The engineered enzyme catalyzes DNA synthesis from DNA as well as RNA templates and enables expeditious quantification of 2′-O-methylation of individual nucleotides directly from total RNA extracts by a simple qRT-PCR.

INTRODUCTION

Modified nucleotides are a ubiquitous feature of life and provide an increased diversity of cellular DNA and RNA. In particular, cellular RNAs contain a large variety of modifications, which are usually introduced by posttranscriptional modification (1,2). To date, more than 100 chemically distinct RNA modifications have been identified in Bacteria, Archaea and Eukarya (3,4). One of the most abundant modifications of RNA is methylation of the 2′-hydroxyl group of the ribose moiety. 2′-O-methylated nucleotides are present in all major classes of eukaryotic RNA, but are best-studied in rRNA. In the human ribosome more than 100 2′-O-methylation sites have been mapped (5,6). Site specific 2′-O-methylation of eukaryotic rRNA is mainly guided by numerous small nucleolar RNAs (snoRNAs), which direct the enzymatic machinery required for methylation toward complementary target regions in the ribosome (7). Those modification sites are highly conserved among vertebrates and mainly occur clustered in functionally important regions, where they are very likely to modulate bio genesis and activity of the ribosome (8). However, the detailed function of 2′-O-methylation in rRNA is not yet well understood. Modifications may fine-tune rRNA folding and a wide range of RNA–RNA and RNA–protein interactions by enhancing hydrophobic surfaces and stabilizing helical stem structures (7). Differential methylation patterns of ribosomes are proposed to be a potential source of heterogeneity that may confer regulatory control of translation through ‘specialized ribosomes’ (9). The fact that alterations or defects in ribosomal methylation are associated with heritable diseases and cancer leaves little doubt about its functional significance (10–12). The relevance of RNA-guided 2′-O-methylation of RNAs was further emphasized by the detection of snoRNAs that target other cellular RNAs, including snRNA, tRNAs and possibly even mRNAs for methylation (1,7,13,14). These findings revealed functional roles of 2′-O-methylation in splicing control (15) and further fundamental cellular processes. Evidently, many aspects of ribose methylation in RNAs still remain to be discovered. Methods to detect 2′-O-methylation in RNA utilize TLC, HPLC or LC-MS to analyze appropriately labeled RNAs after digestion with suitable endonucleases (16–18). These methods require large amounts of highly pure RNA and are very laborious and not suited to detect modifications in low-abundance RNAs. Different approaches exploit enhanced resistance toward alkaline hydrolysis (19) or differential enzymatic turn-over (20,21) of 2′-O-methylated RNA. For instance, the propensity of some reverse transcription to pause primer elongation at low dNTP concentrations when encountering 2′-O-methylated in the template has been used to find these modifications (22). Those methods do not require purification of RNA species and can be directly applied to total RNA extracts. However, the high labor intensity and, in some cases, the ambiguity of results still hamper RNA methylation analysis (23). We here-with present a conceptually new approach for a significantly simplified RNA methylation analysis. Our studies reveal a thermostable DNA polymerase which is able to utilize RNA "

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as well as DNA as template for DNA synthesis (24) and is able to discriminate 2′-O-methylated from unmethylated RNA. Based on these findings, we generated a DNA polymerase with improved discrimination properties and employed it in a methylation specific direct qRT-PCR assay. Along these lines, we were able to quantify the methylation status of five known methylation sites in human 18s rRNA from a variety of different human cancer cell lines by a simple one-step assay directly applied on total RNA extracts. These findings significantly reduce the complexity of 2′-O-methylation analysis. Our data also provide evidence for the occurrence of some ribosomal heterogeneity within human cell lines.

MATERIALS AND METHODS

Oligonucleotides and human total RNA

DNA Oligonucleotides were purchased from Biomers and were used directly for RT-PCR experiments, or purified by preparative denaturing PAGE for primer extension experiments. Radioactive labeling of primer strands was performed using [γ-32P]-ATP and T4 polynucleotide kinase according to the vendor’s protocol. RNA oligonucleotides were purchased from Purimex. The sequences of all used oligonucleotides are listed in Supplementary Table S1. Extraction of total RNA from human cell lines was achieved using the Direct-zol™ RNA MiniPrep Kit (Zymo Research) according to the vendor’s protocol. In-column DNase treatment was performed by the addition of 20u DNase I per column and incubation at room temperature for 15 min. RNA concentration was determined using NanoDrop™ 1000 Spectrophotometer (PEQLAB). Determined A260/A280 values were ≥2.0. RNA integrity was analyzed by agarose gel electrophoresis and the 28s/18s rRNA ratio ranged from 1.7 to 2.1 (Supplementary Figure S1).

In vitro transcribed RNA

cDNA of the human 18s rRNA was obtained by reverse transcription from human RNA extracts using a suitable primer and M-MuLV Reverse Transcriptase (NEB) according to the vendor’s protocol, and amplified by PCR employing Phusion® DNA polymerase (NEB). Amplified cDNA was purified via agarose gel electrophoresis. cDNA was cloned into a pJET 1.2 vector (Thermo), transformed into E.coli BL21 DE3 (Novagen) as described (24). Purification of 6x His-tagged KlenTaq variants was achieved via heat denaturation of lysates at 75°C for 45 min, followed by ultracentrifugation at 20,000 rpm for 45 min and FPLC purification using a His-Trap FF crude column (GE HEALTHCARE) and a linear gradient from 5 to 500 mM imidazole (binding buffer: 100 mM Trizma® base (pH 9.2), 5 mM MgCl2, 300 mM NaCl, 5 mM Imidazol; elution buffer: 100 mM Trizma® base (pH 9.2), 5 mM MgCl2, 300 mM NaCl, 500 mM Imidazol). Enzymes were then concentrated by Vivaspin (Sartorius) and stored in storage buffer (50 mM Trizma base (pH 9.2), 2.5 mM MgCl2, 16 mM (NH4)2SO4, 0.1% (v/v) Tween, 50% (v/v) Glycerol) at −20°C. Purity of enzymes was validated by SDS-PAGE (Supplementary Figure S2).

Protein expression and purification

Protein expression was performed in E.coli BL21 DE3 (Novagen) as described (24). Purification of 6x His-tagged KlenTaq variants was achieved via heat denaturation of lysates at 75°C for 45 min, followed by ultracentrifugation at 20,000 rpm for 45 min and FPLC purification using a His-Trap FF crude column (GE HEALTHCARE) and a linear gradient from 5 to 500 mM imidazole (binding buffer: 100 mM Trizma® base (pH 9.2), 5 mM MgCl2, 300 mM NaCl, 5 mM Imidazol; elution buffer: 100 mM Trizma® base (pH 9.2), 5 mM MgCl2, 300 mM NaCl, 500 mM Imidazol). Enzymes were then concentrated by Vivaspin (Sartorius) and stored in storage buffer (50 mM Trizma base (pH 9.2), 2.5 mM MgCl2, 16 mM (NH4)2SO4, 0.1% (v/v) Tween, 50% (v/v) Glycerol) at −20°C. Purity of enzymes was validated by SDS-PAGE (Supplementary Figure S2).

RT-KTQ-LSIM library construction

All possible RT-KTQ-LSIM single mutants at the positions G668, V669, G672, R746, K747 and N750 were created by site directed mutagenesis of the respective codon. To obtain all 19 mutants at one site, 19 separate PCR reactions were performed respectively, each with the same 5′-phosphorylated reverse primer, but with an individual forward primer carrying the triplet coding for the destined amino acid. After PCR amplification employing Phusion® DNA polymerase and DpnI digestion of the template plasmid, reactions were purified using a NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). PCR products were ligated using T4 DNA ligase (NEB) and transformed into E.coli BL21 DE3 (Novagen). Plasmids were sequenced by Sanger sequencing (GATC Biotech) and clones carrying plasmids with correct RT-KTQ-LSIM mutants were cultured overnight in 700 µL LB-medium containing 100 µg/ml carbenicillin in 96 well deep-well plates at 37°C. Subsequently, 700 µl of 60% (v/v) glycerol in LB-medium was added and plates were stored at −80°C.

Screening for improved RT-KTQ mutants

RT-KTQ-LSIM variants were expressed in duplicates in 96 well plates. Harvested cells were resuspended in 1x KlenTaq reaction buffer (50 mM Trizma® base (pH 9.2), 16 mM (NH4)2SO4, 2.5 mM MgCl2, 0.1% (v/v) Tween) containing 0.5 mg/ml lysozyme, and lysed by incubation at 37°C for 20 min. After denaturation of E.coli host proteins by incubation at 75°C for 45 min, plates were centrifuged at 4400 rpm and 4°C for 30 min and lysates were directly deployed in qRT-PCR (25). For this purpose, 10 µl lysate was mixed with 10 µl 2x Mastermix (400 µM dNTPs, 200 nM forward primer, 200 nM reverse primer, 100 mM betaine, 2x SYBR® green I (sigma), 100 nM Taq DNA polymerase.

was determined using NanoDrop™ 1000 Spectrophotometer (PEQLAB). A260/A280 was 2.4. Purity of the in vitro transcribed RNA was additionally analyzed by agarose gel electrophoresis (Supplementary Figure S1). For later usage, RNA was stored at −80°C.
aptamer (26) and 200 pM RNA template in 1x KlenTaq reaction buffer and analyzed by qRT-PCR, using a Roche Lightcycler® 96 instrument with the following protocol: 60 s at 95°C; then 50 cycles of 15 s at 95°C and 30 s at 62°C. RT-KTQ-LSIM variants with improved discrimination of 2'-O-methylated and unmethylated template were expressed in a larger scale and purified before further characterization.

Primer extension assay with RNA oligonucleotides as template

The reaction mixture contained 150 nM of [γ-32P]-labeled primer, 225 nM of the respective RNA template and 200 μM dNTPs (each) in 1x KlenTaq reaction buffer. Reaction mixtures (20 μL, respectively) were heated to 95°C for 2 min and subsequently cooled to 55°C. After starting the primer extension by addition of either 250 pM RT-KTQ-LSIM or 2.5 nM RT-KTQ-LSIM V669L, reactions were allowed to proceed at 55°C for 10 min. Reactions were stopped by addition of 40 μL stop solution (80% (v/v) formamide, 20 mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) and analyzed by 12% denaturing PAGE. Visualization was performed by phosphorimaging.

Specific Activity of RT-KTQ variants

Extension of primer in complex with the respective RNA template was performed as described above, analyzed by 12% denaturing PAGE and visualized via phosphorimaging. Reactions were performed with various amounts of DNA polymerase (RT-KTQ-LSIM: 0.25–100 fmol; RT-KTQ-LSIM V669L: 5–300 fmol) in 20 μL reaction mixture. The observed intensities of each band in the autoradiogram yielded the conversion of dNTPs in each reaction. dNTP conversion per min was plotted against the amount of applied enzyme. The linear range was analyzed and slopes were obtained by linear regression (Supplementary Figure S3), yielding the specific activity of the respective enzyme and the respective sequence context (27).

Primer extension assay with human RNA extracts/in vitro transcribed 18s rRNA as template

The reaction mixture contained 30 nM of [γ-32P]-labeled primer, 200 μM dNTPs (each) and 200 ng/μl total RNA extracted 18s rRNA in 1x KlenTaq reaction buffer. Reaction mixtures (20 μL, respectively) were heated to 95°C for 2 min and subsequently cooled to 55°C. After starting the primer extension by addition of 20 nM RT-KTQ-LSIM V669L, reactions were allowed to proceed at 55°C for 10 min. Reactions were stopped by addition of 40 μL stop solution (80% (v/v) formamide, 20 mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) and analyzed by 12% denaturing PAGE. Visualization was performed by phosphorimaging.

qRT-PCR with human RNA extracts and in vitro transcribed 18s rRNA

qRT-PCRs were conducted in a total volume of 20 μl reaction mixture containing 100 nM forward primer, 100 nM reverse primer, 200 μM dNTPs (each), 0.5 M betaine, 1x SYBR® green I (sigma), 100 nM Taq Aptamer (26) and 100 nM of either RT-KTQ-LSIM or RT-KTQ-LSIM V669L in KlenTaq reaction buffer. qRT-PCR was conducted in triplicates using a Roche Lightcycler® 96 instrument with the following protocol: 60 s at 95°C; then 40 cycles of 15 s at 95°C and 30 s at 62°C. For Analysis of the RT-PCR by Agarose Gel electrophoresis, qRT-PCR was stopped after 25 (RNA template A and 2’Omec) or 30 cycles (RNA template C and 2’Omec), respectively. ΔCt-values were used to calculate 2’-O-methylation fractions. Efficiency of the PCR was determined by template dilution series using the unmodified templates.
extension of a primer hybridized to different RNA oligonucleotides with identical sequence but carrying either a 2′-O-methylated nucleotide at the position of first incorporation or the respective unmodified one (Figure 1). We found that 2′-O-methylation is a major obstacle for reverse transcription catalyzed by RT-KTQ-LSIM even at dNTP concentrations of 200 µM and irrespective of the methylated nucleotide carrying a pyrimidine (C) or a purine (A) as nucleobase (Figure 1B). Since RT-KTQ-LSIM is a thermostable enzyme, we envisioned to exploit this discrimination in order to develop a methylation specific qRT-PCR system. Therefore, we applied the aforementioned primer/template combination and designed an appropriate forward primer which results in a PCR amplicon of 53 nt. As CT-values of qRT-PCR experiments also depend on the concentration of the target RNA, we designed an additional reverse primer, which terminates several nucleotides downstream of the analyzed nucleobase, as a control (Figure 1C). Using this control reverse primer in combination with the same forward primer, should result in a qRT-PCR that is not impeded by methylation of the respective nucleotide. When using the methylation-specific primers, qRT-PCR did indeed result in a delayed amplification of methylated RNAs in comparison to the respective unmethylated ones, while amplification curves coincided when using the control primer sets. The discrimination was higher for a methylated cytidine in the template (average ΔCT = 4.6) than for a methylated adenosine (average ΔCT = 1.9).

**Generation of an improved RT-KTQ variant**

We aimed to generate RT-KTQ-LSIM variants with improved discrimination of 2′-O-methylated nucleotides. Therefore, we created libraries with single mutants of RT-KTQ-LSIM by site-directed mutagenesis. Mutation sites were selected by inspection of the crystal structure of RT-KTQ-LSIM in complex with DNA/RNA hybrid and a bound triphosphate (24) (Figure 2). Amino acids with close proximity to the 2′-O-hydroxyl of the ribonucleotide paired to the incoming dNTP (namely G668, V669, G672, R746, K747 and N750) were chosen for saturation mutagenesis. Libraries were screened for improved discrimination of 2′OmeA from unmethylated A directly from cell lysate (25), using the above-named qRT-PCR system. Mutants which exhibited increased ΔCT-values between the respective templates were Ni-NTA purified and further analyzed by primer extension and qRT-PCR experiments. Best results were obtained with an RT-KTQ-LSIM V669L mutant featuring enhanced discrimination of 2′-O-methylated RNA in both, primer extension and qRT-PCR (Figure 3A–C). The ΔCT-value did not only increase for the screened 2′OmeA/A discrimination (average ΔCT = 7.7), but also when comparing the methylated C template to the unmethylated one (average ΔCT = 6.3). By looking at the specific activity (27) of RT-KTQ-LSIM V669L and RT-KTQ-LSIM on unmethylated and methylated RNA templates, the improved properties of the mutant become evident. Although both enzymes exhibit differences between activities on methylated and unmethylated templates, these differences raise from ~3.5-fold for RT-KTQ-LSIM to ~7-fold for RT-KTQ-LSIM V669L (Table 1 and Supplementary Figure S3). Moreover, the ability of RT-KTQ-LSIM V669L to utilize these catalytic differences in qRT-PCR is enhanced due to its decreased activity on RNA, resulting in an increased limitation of the actual discriminating step, namely first strand synthesis by reverse transcription.

**Direct quantification of RNA 2′-O-methylation by qRT-PCR**

We hypothesized that qRT-PCR, catalyzed by RT-KTQ-LSIM V669L, could be evaluated in a quantitative manner to determine the fraction of 2′-O-methylation at a target site. Therefore, we mixed known ratios of a 2′-O-methylated RNA template with its unmethylated equivalent and measured ΔCT values in reference to the fully unmethylated template. By approximation, we postulated that any amplification of the target derives from the unmethylated template only, enabling the calculation of the methylation fraction by the comparative CT method (28):

\[
\text{methylation fraction} = 1 - E^{-\left(\Delta CT_{\text{methylation specific primers}} - \Delta CT_{\text{control primers}}\right)}
\]

*with E = PCR efficiency*

This seems to be a valid assumption as a ΔCT value of ~7 cycles, as caused by RNA methylation, corresponds to a decrease of RNA concentration by two orders of magnitude, resulting in an error of only ~1%. Our data confirm that this approach actually allows a very accurate estimation of the methylation fraction (Figure 3D). Next, we used this direct qRT-PCR assay to analyze the methylation fraction of five known methylation sites (namely A27, A99, U428, G1490 and C1703) in human 18s rRNA directly from total RNA extracts deriving from various immortalized and/or cancer cell lines. As unmodified reference RNA, we employed in vitro transcribed 18s rRNA. For each site, RNA concentrations were adjusted by qRT-PCR with a control primer terminating 5 or 6 nucleotides downstream of the methylation site. To analyze the methylation site, reverse primers were designed which directly terminate one nucleotide upstream of the analyzed nucleotide. Both reverse primers were combined with the same forward primer, delivering amplicons of ~40–60 nt, which contain only one site of methylation (cf. Figure 1C). Our results show that most of the analyzed sites are methylated uniformly throughout different cell lines and methylation fractions range from 80% upward (Figure 4A, Supplementary Table S3). The methylation of A99, however, drops below 50% in colorectal cancer cells (Caco2) (Figure 4B). Inhomogeneity of A99 methylation was further verified by radioactive primer extension experiments catalyzed by RT-KTQ-LSIM V669L (Supplementary Figure S5).

**DISCUSSION**

In summary, we found that DNA synthesis by a KlenTaq DNA polymerase variant with reverse transcriptase activity is stalled by the presence of 2′-O-methylation in the RNA template and employed this effect in a methylation-sensitive qRT-PCR. These findings pave the way for a significantly
Figure 1. DNA synthesis catalyzed by RT-KTQ-LSIM is hampered by 2'-O-methylation of RNA templates. (A) Structures of relevant nucleotides. (B) Primer extension in presence of methylated or unmethylated RNA templates catalyzed by RT-KTQ-LSIM. (C) qRT-PCR amplification of methylated and unmethylated RNA oligonucleotides catalyzed by RT-KTQ-LSIM.

Table 1. Specific activity of RT-KTQ-LSIM variants on methylated and unmethylated templates

| DNA polymerase             | template  | specific activity [min⁻¹] |
|----------------------------|-----------|--------------------------|
| RT-KTQ-LSIM                | A         | 94.9 ± 1.8               |
| RT-KTQ-LSIM                | 2'OmeA    | 30.5 ± 1.6               |
| RT-KTQ-LSIM                | C         | 120.9 ± 7.9              |
| RT-KTQ-LSIM                | 2'OmeC    | 25.6 ± 1.1               |
| RT-KTQ-LSIM V669L          | A         | 22.1 ± 1.3               |
| RT-KTQ-LSIM V669L          | 2'OmeA    | 3.17 ± 0.07              |
| RT-KTQ-LSIM V669L          | C         | 25.6 ± 1.1               |
| RT-KTQ-LSIM V669L          | 2'OmeC    | 3.83 ± 0.08              |

Data points derive from triplicates. ± describes SD.
Figure 2. Rational design of RT-KTQ-LSIM libraries. Amino acids in immediate proximity to the 2'-oxygen of the nucleotide paired to the incoming dNTP were selected for saturation mutagenesis (namely G668, V669, G672, R746, K747 and N750). Adapted from PDB 4BWM (24) using PyMOL (Schrödinger, LLC, New York, NY, USA).

Figure 3. RT-KTQ-LSIM V669L features increased discrimination between 2'-O-methylated and unmethylated RNA templates and enables quantification of 2'-O-methylation by qRT-PCR. (A) Primer extension in the presence of methylated or unmethylated RNA templates catalyzed by RT-KTQ-LSIM V669L. (B) qRT-PCR amplification of methylated and unmethylated RNA oligonucleotides catalyzed by RT-KTQ-LSIM V669L. (C) RT-PCR reactions were stopped after 25 cycles (top) or 30 cycles (bottom) and analyzed by agarose gel electrophoresis. (D) The ΔCt-method was used to calculate methylation ratio of RNA template at 100 pM concentration with varied fractions of 2’OmeA/A at the target position. Error bars describe SD (n = 3).
engineered and screening of new DNA polymerase variants may open up this qRT-PCR-based approach for analysis of other biologically significant RNA modifications (33) in the future.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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