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Cycloaddition enabled mutational profiling of 5-vinyluridine in RNA

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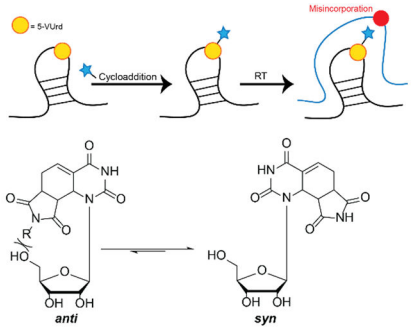
Abstract

We report the detection of 5-vinyluridine (5-VUrd) in RNA at single nucleotide resolution via mutational profiling. Maleimide cycloadducts with 5-VUrd in RNA cause a stop in primer extension during reverse transcription, and the full-length cDNA product from reverse transcription contains misincorporation across the cycloadduct site.

Graphical Abstract



Cellular responses to external stimuli rely on subtle changes in tightly regulated gene expression networks, and the study of these changes provides molecular insights into processes such as disease progression and cellular differentiation. Although changes in protein expression in response to various stimuli have been widely studied, few methods exist to study the changes in RNA expression as cellular response mechanisms. Advances in next-generation sequencing have provided facile access to the study of RNA expression; however, RNA sequencing alone is unable to report on the dynamics of RNA expression and cannot distinguish between synthesis and degradation rates of RNA which is crucial for

Conflicts of interest

The authors declare no competing financial interest.

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elucidating the dynamic molecular mechanisms that regulate gene expression.[1,2] Therefore, methods to differentiate nascent RNA from total RNA are essential for identifying changes in the synthesis and degradation rates of RNA in response to external stimuli.

RNA metabolic labeling has been used to study nascent RNA transcripts, where an unnatural nucleoside can be incorporated into the actively transcribed RNA by the endogenous pathways of the cells.[3–6] Once the modified nucleoside is incorporated into the RNA, chemical conjugation can be performed to attach an affinity tag onto nascent RNA and the transcripts can be enriched from the pool of total RNA. However, enrichment protocols can often be laborious and provide variable yields that complicate RNA expression analysis. Therefore, some methods to profile nascent RNA transcripts without enrichment have been developed, where unnatural nucleosides can be chemically modified to induce mutations (mutational profiling) upon reverse transcription (RT). Examples of such methods include SLAM seq, TUC seq, and TimeLapse seq, which rely on the metabolic incorporation of 4-thiouridine (4-SUrd) by cells into nascent RNA.[7–9] Recently, 5-ethynyluridine (5-EUrd) in nascent RNA was detected after RNA metabolic labeling using direct RNA sequencing with nanopore sequencing platform without requiring any chemical modification of the RNA.[10] However, both 4-SUrd and 5-EUrd have been shown to have cytotoxicity issues with biological systems,[11,12] necessitating the development of sequencing nascent RNA without cytotoxicity concerns and enrichment protocols.

Vinyl nucleosides can be incorporated into nascent RNA by the endogenous nucleoside salvage pathways of the cells. Notably, it was observed that 5-vinyluridine (5-VUrd) can be incorporated at a similar rate as other uridine analogues, showed minimal toxic effects and transcriptional changes in cells when compared to the widely used 5-EUrd.[3] Further, the nucleobase 5-vinyluracil can be utilized to incorporate 5-VUrd in a cell-type-specific manner, where only cells expressing an optimized uracil phosphoribosyl transferase can incorporate 5-VUrd in their RNA.[13,14] Therefore, 5-VUrd presents a non-toxic alternative for use in *in-vivo* RNA metabolic labeling and nascent RNA sequencing. However, there are no methods available to utilize this modified nucleoside for RNA expression profiling using mutation profiling, as described above. Here, we report the detection of 5-VUrd in an *in-vitro* transcribed RNA with mutational profiling (Figure 1A).

Several chemical adducts on RNA have been shown to induce stalling of RT during primer extension (RT stop) and misincorporations across the modified sites in the full-length cDNA, facilitating mutational profiling of the adducts.[15–17] 5-VUrd has been demonstrated by our group to react with maleimide derivatives in a *[4+2]* cycloaddition reaction, and we envisioned these cycloadducts undergoing an *anti* to *syn* conformational change to reduce steric clash with 5′ OH of the sugar. This conformational change would expose the non- Watson-Crick-Franklin (WCF) face of the nucleobase to the RT enzyme during primer extension, which might not be recognized by the enzyme and lead to RT stop or misincorporation across the cycloadduct (Figure 1B). In this scenario, substitutions on the nitrogen of the maleimide could dictate the efficiency of RT stop and mutational profiling. Therefore, we began by evaluating the reactivity of 5-VUrd with maleimides having substitutions of varying size on the maleimide nitrogen, ranging from methyl to 4-bromophenyl substituent. The maleimides were incubated with 5-VUrd and conversion

was monitored via ^{1}H NMR spectroscopy. Moderate to high conversions were observed with all maleimides containing substitutions on the nitrogen (Figure 2). This substrate scope suggested that all the substituted maleimides could be used for reactions with 5-VUrd containing RNA to test for the effect of cycloadduct size on RT.

To study the effect of cycloadduct formation on 5-VUrd-containing RNA on RT extension, we designed a DNA template for *in-vitro* transcription (IVT) of RNA containing a single adenosine to direct the insertion of a single uridine or 5-VUrd in the RNA synthesized upon transcription (Figure 3A). By modifying nucleotide triphosphate mixtures, we performed in-vitro transcription to synthesize RNA transcripts containing either 5-VUrd (**T1**) or uridine (**T2**) with the use of 5-vinyluridine triphosphate or uridine triphosphate respectively (Figure 3B). To check for the successful incorporation of 5-VUrd in RNA, both transcripts were treated with maleimide-biotin and detected biotinylation via streptavidin-conjugated horseradish peroxidase to visualize chemiluminescence with luminol on a dot-blot assay (Figure 3C). As expected, chemiluminescence was observed upon the reaction of **T1** with both maleimide-biotin on the dot-blot, whereas **T2** did not show significant biotinylation (Figure 3D).

With these transcripts in hand, we investigated the effect of maleimide conjugation on RT with a primer extension assay. **T1** was reacted with the maleimides **1**-**6**, and RT reactions were performed with a murine leukemia viral RT enzyme (SuperScript III). We observed RT stop at one nucleotide before the modified 5-VUrd position (Figure 4A). RT stop was not significant with unsubstituted maleimide 1 and less bulky maleimide 2; however, significant truncation of cDNA was observed with maleimides **3**, **4**, **5**, and **6**, suggesting that the RT enzyme experiences a steric clash from the non-Watson-Crick-Franklin (non-WCF) face of the nucleobase in primer extension upon encountering the modified nucleoside. Comparing the benzyl-substituted maleimide **4** with the phenyl-substituted maleimides **3**, **5**, and **6**, modification on T1 with 4 resulted in a lower RT stop. This may be attributed to the flexibility of the methylene unit between the maleimide and aromatic ring, which could provide less hindrance for the RT enzyme compared to rigid phenyl substituted maleimides. To further quantify the yield of full-length cDNA in the RT reactions upon maleimide conjugation, we performed qPCR with cDNA present in the RT reactions with primers designed to selectively amplify only the full-length cDNA. As seen with the primer extension assay, the full-length cDNA yield of **T1** with **1**, **2** and **4** was comparable to DMSO treated samples, whereas yields were significantly reduced upon the reaction of **T1** with **3**, **5** and **6** (Figure 4B). Overall, the qPCR data and the primer extension assay results demonstrate the ability of N-substituted maleimides to detect 5-VUrd via RT stalling in primer extension assays.

With the observed RT stop after maleimide conjugation, we decided to study the nucleotide incorporation pattern across the cycloadduct by various RT enzymes to develop a mutational profiling approach for detecting 5-VUrd in RNA. We chose to modify **T1** with **1** and **3** for the RT reactions, where RT of **T1** treated with **1** did not produce a significant RT stop whereas RT of **T1** treated with **3** produced a significant RT stop. We chose a few viral RT enzymes, including HIV-RT which is known to incorporate mutations across a modified

nucleobase,[18] and tried using Mn^{+2} instead of Mg^{+2} in the RT buffer to identify the RT conditions that optimize misincorporation rates across from the 5-VUrd cycloadduct.[15]

Initially, we performed a primer extension assay with the different RT conditions, and we observed similar RT stops with all the RT reaction conditions (Figure S1). To study nucleotide incorporation across the cycloadduct site, the full-length cDNA from the RT reactions was selectively amplified and converted to dsDNA for subsequent sequencing. Upon alignment of reads to the sense strand from dsDNA of **T2** (reference strand, Figure 5A), nucleotide reads at the 5-VUrd position from dsDNA of **T1** (mapped strand, Figure 5A) were analyzed to observe misincorporation across the cycloadduct site. We observed a high level of misincorporation across the cycloadduct site in **T1** treated with **3** when RT was performed with SuperScript II in Mn^{+2} buffer compared to treatment with **1** and DMSO (Figure 5B). Various nucleotide misincorporation patterns were observed with the different RT conditions performed on **T1** treated with **3** (Figure S2), however; with RT in Mn^{+2} buffer, >80% of the mutations were T->A mutations, implying that the RT enzyme primarily misidentified the cycloadduct as adenosine (Figure S3). Additionally, these same RT conditions when performed with **T2** transcript treated with either **1**, **3,** or DMSO did not observe any significant mutation across the uridine in any condition (Figure S4).

Although the *syn* cycloadduct cannot form hydrogen bonding interactions to explain the incorporation of thymidine across the modified site, we believe thymidine may be incorporated across the cycloadduct site to compensate for the increase in size compared to uridine and 5-VUrd. With this reasoning, we looked for differences in the mutational frequency of **T1** reacted with various maleimides and sequenced full-length cDNA after performing RT with SuperScript II in Mn^{+2} buffer. We observed higher mutations with maleimides that resulted in higher RT stop, suggesting that increasing steric hindrance to the RT enzyme with increasing size of the cycloadduct led to more misincorporation (Figure 5C). Interestingly, all maleimides resulted in most mutations being T->A; however, maleimides **2**, **3**, **4**, **5** and **6** had > 80% T->A mutation, significantly higher compared to DMSO negative control (Figure 5D). Overall, these results show the potential of mutational profiling to track the incorporation of 5-VUrd in RNA metabolic labeling experiments, where transcripts that yield significantly higher T->A mutations compared to untreated samples upon RNA sequencing can be identified as nascent transcripts without pre-enrichment from the total RNA pool.

The development of novel yet easy-to-perform methods for tracking nascent RNA synthesis in cells is a critical tool for investigations into cellular response mechanisms. In this study, we developed a mutational profiling approach to detect 5-VUrd in RNA with maleimide cycloadduct formation. Based on mutational and RT stop frequencies observed in our studies, we believe maleimides **2** and **3** would be a balanced reagents for optimizing a mutational profiling approach with total RNA from cellular samples as they deliver significant mutational frequency with moderate full-length cDNA synthesis. We are currently working on optimizing and extending the use 5-VUrd for probing changes in RNA expression of cells upon various external stimuli such as heat shock or drug administration, where cells can be treated with 5-VUrd to label nascent RNA transcripts after stimuli exposure. These transcripts can then be recovered from the cells at various intervals and

changes in RNA expression can be observed by mutational profiling of 5-VUrd after RT in optimized conditions. Further, we anticipate our novel methodology to be amenable to cell-specific RNA metabolic labelling *in-vivo*[13,14] where we have detailed the use of 5-VUrd using conventional methods and will benefit from our approach detailed herein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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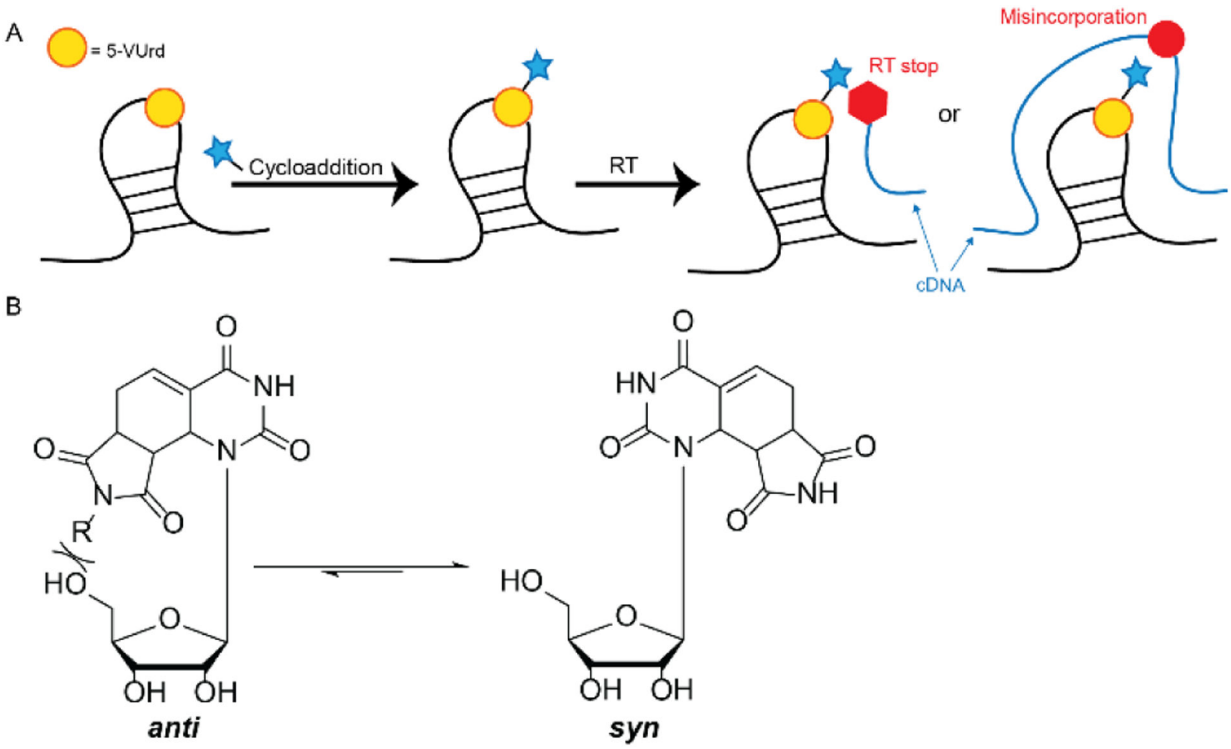

Figure 1.

Mutational profiling for RNA metabolic labeling analysis. A) Detection of 5-VUrd in RNA with RT stop and mutational profiling. B) Proposed *anti* to *syn* conformational change of 5-VUrd maleimide cycloadduct.

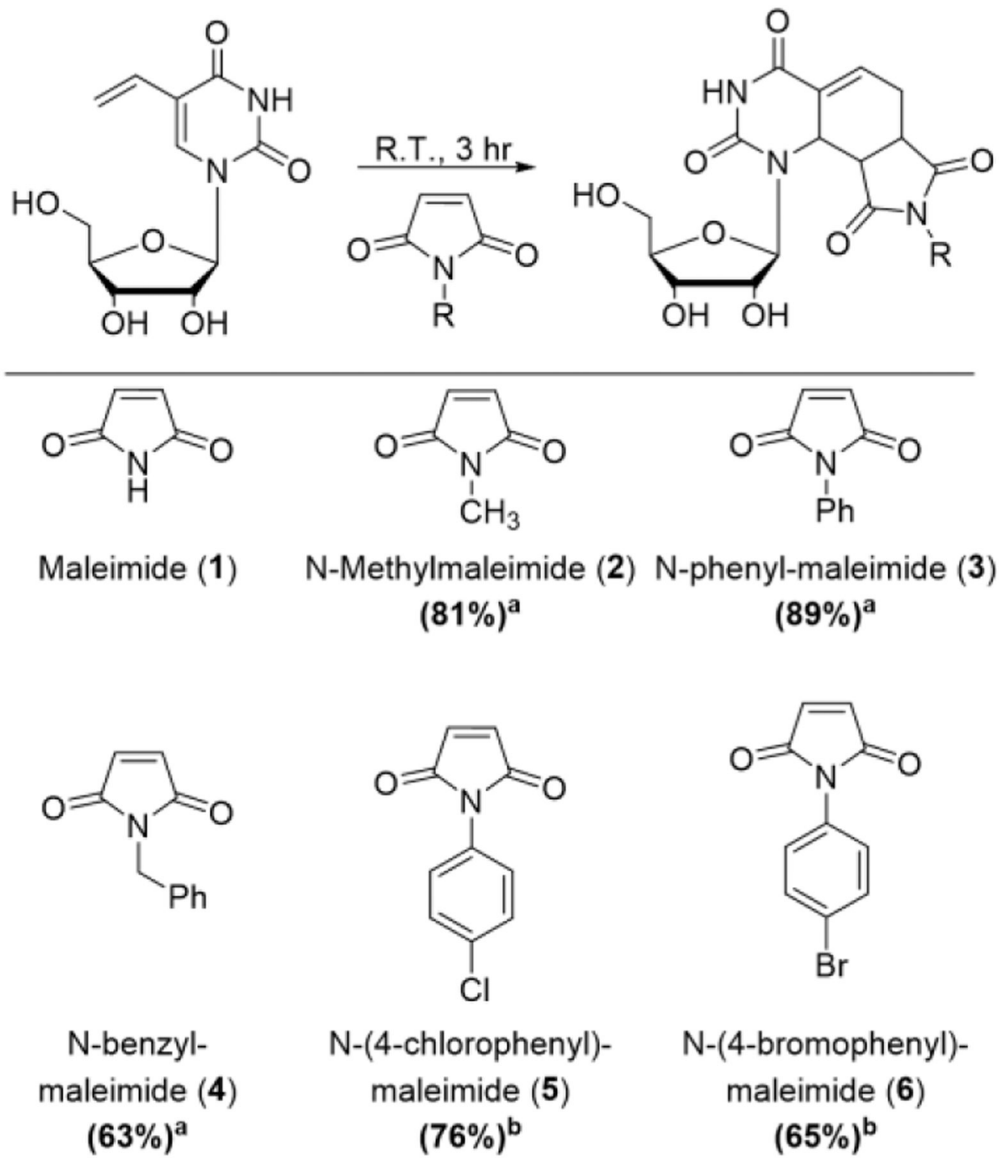

Figure 2.
Maleimide substrate scope study with 5-VUrd. [a]D_2O as solvent. [b]DMSO-d_6 as solvent.

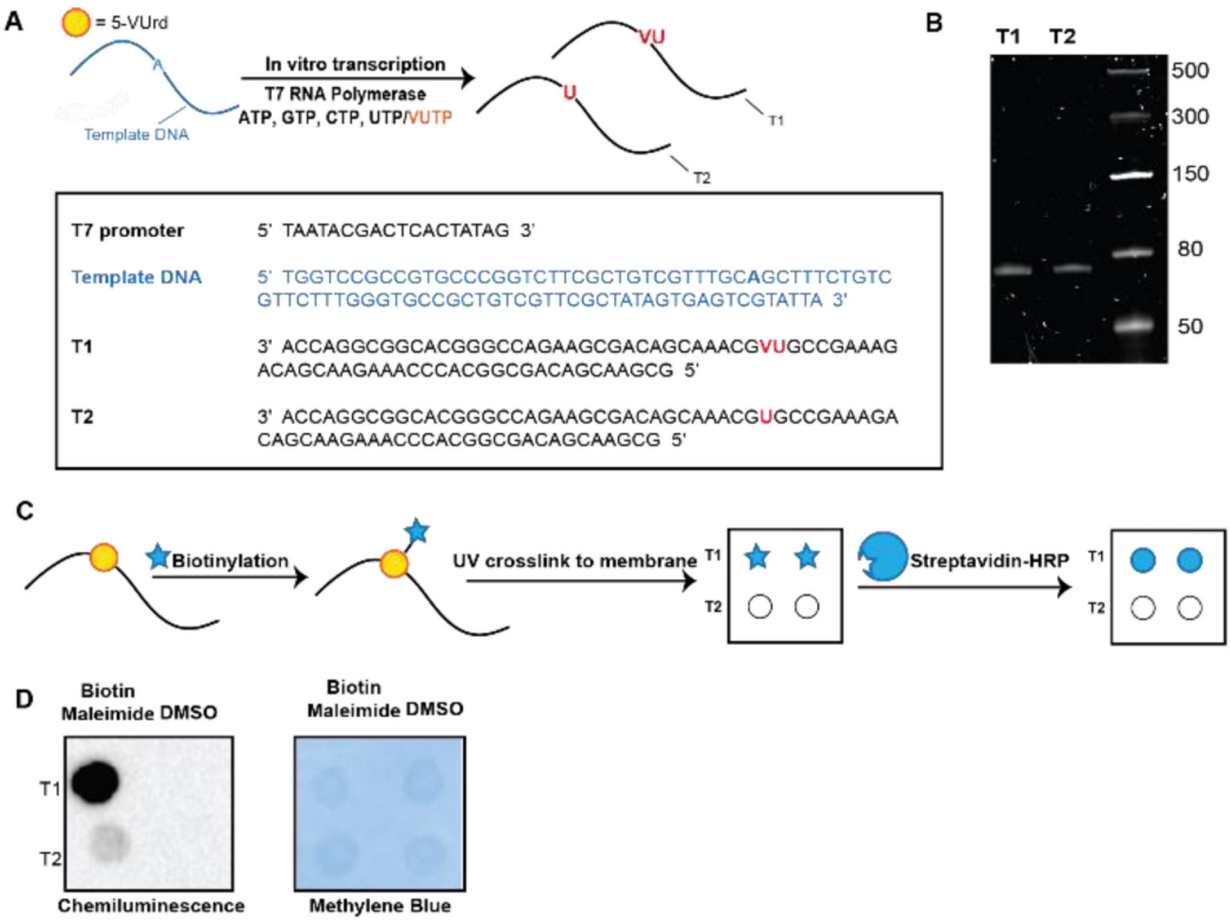

Figure 3.

In-vitro transcription of RNA. A) Double-stranded DNA template design, RNA sequences (with uridine or 5-VUrd highlighted), and scheme for in-vitro transcription. B) Gel electrophoresis to confirm RNA length and integrity. C) Scheme for chemiluminescence dot-blot assay. D) RNA biotinylation and dot-blot to confirm the incorporation of 5-VUrd.

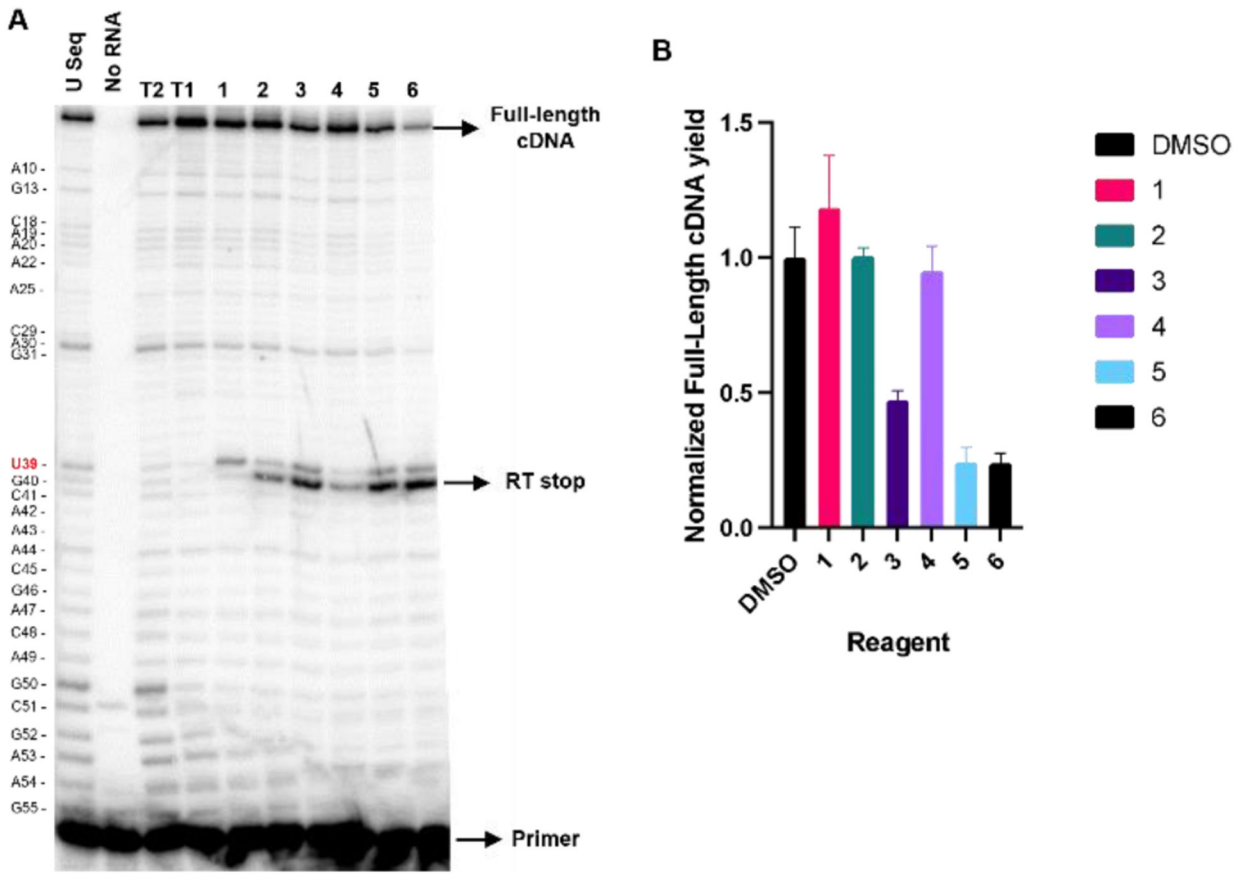

Figure 4.

RT properties of 5-VUrd containing RNA. A) Primer extension assay to observe RT stop with different maleimide substrates. B) Quantification of full-length cDNA production in RT reaction with SuperScript-III via qPCR.

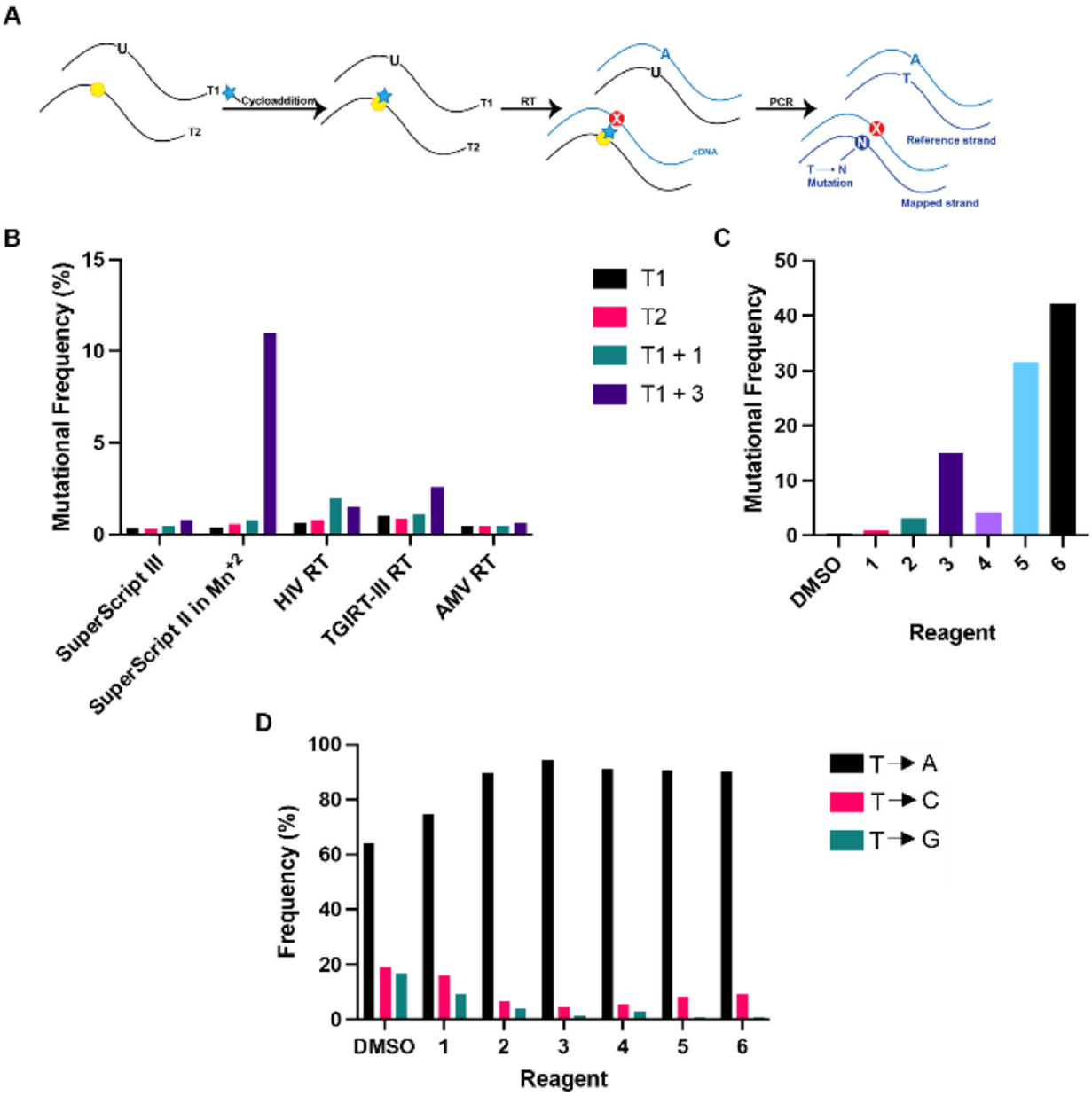

Figure 5.

Mutation of full-length cDNA and misincorporation across 5-VUrd in RNA. A) Mutation frequency in full-length cDNA with various RT conditions. B) Mutational frequency in full-length cDNA with various maleimide substrates. C) Characterization of the misincorporated base with various maleimide substrates across 5-VUrd in T2 with SuperScript II in Mn^{+2} buffer. D) Base identity of the mutated base across 5-VUrd in T2 treated with maleimides 1–6 and RT with SuperScript II in Mn^{+2} buffer.