SuperScript IV Reverse Transcriptase as a better alternative to AMV-based enzymes

Abstract

Reverse transcriptases (RTs) from avian myeloblastosis virus (AMV) and Moloney murine leukemia virus (MMLV) are commonly used in research to synthesize complementary DNA (cDNA) from RNA templates. MMLV RT–based enzymes were traditionally the preferred choice due to several desirable attributes such as lower RNase H activity, ability to synthesize longer cDNAs, and higher fidelity. AMV RT–based enzymes, including engineered AMV RTs such as Invitrogen™ ThermoScript™ RT, were often recommended in applications requiring higher thermostability than the original generation of MMLV-derived RTs could support. Invitrogen™ SuperScript™ IV RT is a new-generation MMLV-based RT that has been engineered for further thermostability and processivity, and as with all enzymes in the Invitrogen™ SuperScript™ family, reduced RNase H activity. Using a variety of assays, this study demonstrates that SuperScript IV RT offers advantages at standard as well as elevated temperatures over cloned AMV and ThermoScript RT, including higher sensitivity, inhibitor tolerance, and ability to work with degraded RNA. Researchers currently using AMV-based RTs in their protocols are encouraged to use SuperScript IV RT as a replacement.

Introduction

RTs are DNA polymerases that synthesize cDNA from an RNA template. They are widely used to study gene expression in research samples, such as tissues and cells, and more recently in single-cell analysis and next-generation sequencing (NGS) applications.

RTs are characterized by several attributes (Table 1). Enzyme processivity describes the number of nucleotides incorporated during a single binding event of an RT to a primed template before dissociation. High processivity is a key enzyme feature and results in additional desirable attributes such as higher efficiency, shorter reaction time, higher tolerance to inhibitors, and higher thermostability. High RT thermostability allows cDNA synthesis to be performed at elevated temperatures (at 50°C or higher), which helps denature regions of strong RNA secondary structure for longer cDNA and higher yields [1,2].

Table 1. Key performance attributes of different RT enzymes.

	SuperScript IV RT	MMLV RT	AMV RT	ThermoScript RT
Processivity	1,500 nt	30 nt	400 nt	400 nt
Reaction temperature	Up to 65°C	Up to 42°C	Up to 55°C	Up to 65°C
Reaction time	10 min	60 min	60 min	60 min
RNase H activity	Not detectable	Medium	High	Low
Relative cDNA yield*	High	Low	Medium	Medium
Fidelity	++	++	+	+

* cDNA yields from samples including challenging or low-purity RNA.

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In addition to DNA polymerase activity, RTs also possess RNase H endonuclease activity that cleaves the RNA template in an RNA–DNA duplex and often limits full-length cDNA synthesis [3]. Engineered RT variants with reduced endonuclease activity, termed H-minus RTs, are often preferred because of improved performance in synthesizing longer cDNA [4–6].

Commercially available RTs used in molecular biology are generally derived from MMLV or AMV. Cloned AMV RT and engineered variants of AMV RT (e.g., ThermoScript RT) were often used when success of RT reactions depended on higher temperatures than wild type MMLV RT could withstand. In their wild type forms, AMV RTs demonstrate higher processivity at approximately 400 nucleotide additions per binding event, compared to 30 nucleotides for MMLV RT [1,2,7,8]. Newer research applications and use of increasingly difficult sample types in molecular biology research demand superior RTs that not only are highly processive and thermostable, but also demonstrate high inhibitor tolerance, high sensitivity, lower error rate, and shorter reaction times.

SuperScript IV RT, the newest member of the SuperScript family, is an engineered MMLV RT designed for superior performance even with challenging RNA samples. SuperScript IV RT has significantly better processivity compared to other MMLV and AMV RTs, as well as increased thermostability, resistance to inhibitors, and sensitivity [9]. As with all enzymes in the SuperScript family, SuperScript IV RT has reduced, undetectable RNase H activity.

In this study we tested the performance of SuperScript IV RT, cloned AMV RT, and ThermoScript RT at standard and high reaction temperatures (up to 65°C) with RNA of low input, suboptimal purity, or poor quality. The data demonstrate that SuperScript IV RT offers better performance than cloned AMV and ThermoScript RTs under the conditions tested.

Materials and methods

Commercial RNA samples

Commercial RNA samples used included HeLa S3 total RNA (component of Ion Total RNA-Seq Kit v2, Cat. No. 4475936), Invitrogen™ Human Placenta Total RNA (Cat. No. AM7950), and Invitrogen™ 0.5–10 Kb RNA Ladder (Cat. No. 15623200).

Generation of degraded RNA

1 µg each of Human Placenta Total RNA and HeLa S3 total RNA were degraded to an RNA integrity number (RIN) of 2.6 and 1, respectively, by addition of $MgCl_2$ to 1 mM final concentration and incubation at 95°C for 15 minutes. RIN was evaluated using an Agilent™ 2100 Bioanalyzer™ system and Agilent™ RNA 6000 Nano Kit.

cDNA synthesis protocol

The Invitrogen™ SuperScript™ IV First-Strand Synthesis System (Cat. No. 18091050), Invitrogen™ Cloned AMV First-Strand cDNA Synthesis Kit (Cat. No. 12328032), and Invitrogen™ ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis (Cat. No. 11146016) were used according to their standard protocols, unless otherwise specified. For all experiments, oligo$(dT)_{20}$ primers were used. To visualize synthesized cDNA using 0.5–10 Kb RNA Ladder as a template, reactions were resolved by alkaline gel electrophoresis and stained with Invitrogen™ SYBR™ Gold Nucleic Acid Gel Stain (Cat. No. S11494).

qPCR

qPCR was performed on the reverse transcription samples using Thermo Scientific™ Luminaris™ Probe qPCR Master Mix, Low ROX (Cat. No. K0944) with the Applied Biosystems™ TaqMan® Assays indicated in the figures. Reverse transcription reactions made up 10% of the total 20 µL qPCR reaction volume. Assays were performed on an Applied Biosystems™ 7500 Fast Real-Time PCR System. For the inhibitor assays, ΔC_t was calculated as (C_t inhibitor – C_t control).

Endpoint PCR

The reverse transcription reactions contributed 4% of the total PCR reaction volume. Invitrogen™ Platinum™ *Taq* DNA Polymerase High Fidelity (Cat. No. 11304011) was used for PCR amplification using its recommended protocol on an Applied Biosystems™ ProFlex™ PCR System. 8 µL of PCR reaction was resolved using agarose gel electrophoresis in TAE buffer and visualized by ethidium bromide staining.

Results and discussion

Ability to work at increased temperatures

Thermostable RT enzymes allow reactions to be performed at higher temperatures, which helps denature regions of strong RNA secondary structure that can otherwise cause RTs to stall and limit cDNA size. High RT thermostability is a consequence of high processivity, which in turn allows synthesis of long, full-length cDNA in less time [1]. The thermostability of RTs and their ability to synthesize full-length cDNA were compared using 1 µg of 0.5–10 Kb RNA Ladder template with oligo(dT)$_{20}$ primers at temperatures between 45 and 65°C. SuperScript IV RT was tested at reaction times of 10, 30, and 60 minutes (10 minutes is the recommended reaction time), while cloned AMV and ThermoScript RTs were tested at 30 or 60 minutes.

Figure 1 shows that SuperScript IV RT synthesized up to 10 kb cDNA in only 10 minutes, even at 65°C. ThermoScript RT was able to synthesize the largest cDNA fragment at 60 minutes at all temperatures, while cloned AMV RT was able to synthesize up to 10 kb cDNA in 60 minutes at 60°C, but only up to 6 kb at 65°C. The same results were observed for the 30-minute reverse transcription reactions (data not shown). These results demonstrate that due to its exceptional processivity and enhanced thermostability, SuperScript IV RT was able to synthesize large, full-length cDNA fragments in 10 minutes, even at challenging reaction temperatures. Other enzymes require more time for cDNA synthesis or don't perform as well at the highest temperatures.

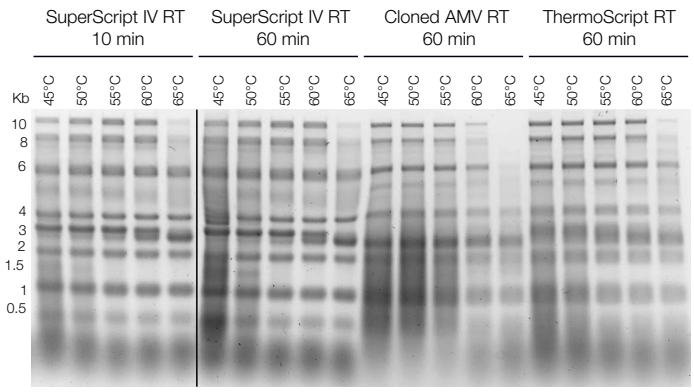

Figure 1. Ability to synthesize cDNAs of different lengths at a range of temperatures and reaction times using SuperScript IV RT, AMV RT, and ThermoScript RT.

Performance in the presence of inhibitors

Numerous compounds that have inhibitory effects on RTs are commonly found in RNA samples, even after employing thorough purification methods [9,10]. Some examples of inhibitory compounds include phenol from Invitrogen™ TRIzol™ Reagent that is used to extract RNA from cells and tissues, and salts such as guanidine isothiocyanate that is used in multiple steps during RNA isolation. RT inhibitors may also be inherent in the biological sample source: hematin from blood, bile salts from blood and feces, and humic acid from soil and thus, from plants.

To test how different compounds affect RT enzymes, cDNA synthesis was performed using 100 ng of total HeLa RNA in the presence of individual inhibitors at final concentrations indicated in Figure 2 in optimal (50°C) as well as challenging (65°C) reaction conditions. At 50°C, the reaction time was the recommended 10 minutes for SuperScript IV RT and 60 minutes for cloned AMV and ThermoScript RTs. At 65°C, the reaction time was 60 minutes for all three enzymes. qPCR was performed with TaqMan Assays for ACTB and GUSB targets.

Figure 2 shows the ΔC_t value between the inhibitor-containing and control RT-qPCR reactions for each sample. In almost all conditions tested, ΔC_t values for SuperScript IV RT were lower than ΔC_t values for cloned AMV and ThermoScript RTs, confirming that SuperScript IV RT has better inhibitor tolerance. The improvement is especially pronounced at the challenging 65°C reaction temperature.

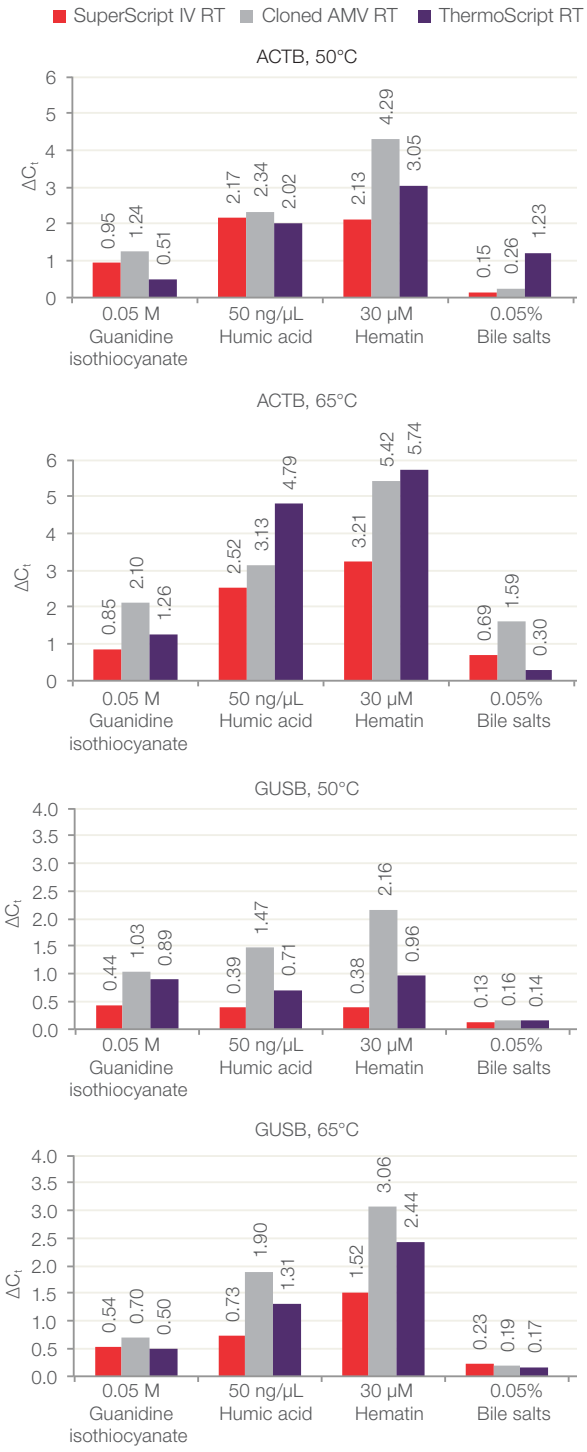

Figure 2. Two-step RT-qPCR performance using SuperScript IV RT, AMV RT, and ThermoScript RT in the presence of inhibitors (ΔC_t = C_t inhibitor – C_t control).

cDNA synthesis sensitivity

High sensitivity, or the ability of RTs to generate cDNA from very little input RNA, is an important attribute of this class of enzymes, especially as researchers increasingly work on smaller RNA input. The sensitivity of the three RTs was tested by performing two-step RT-PCR with 1 µg down to 1 pg of total HeLa RNA input at 50°C and 65°C. At 50°C, the reaction time was 10 minutes for SuperScript IV RT and 60 minutes for AMV and ThermoScript RTs. At 65°C, all three RTs were tested using a 60-minute reaction time. The synthesized cDNA was then amplified by endpoint PCR using Platinum *Taq* DNA Polymerase High Fidelity with primer pairs for ACTB (356 bp), LAM (997 bp), and ACTR (3,009 bp) targets. PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining (Figure 3).

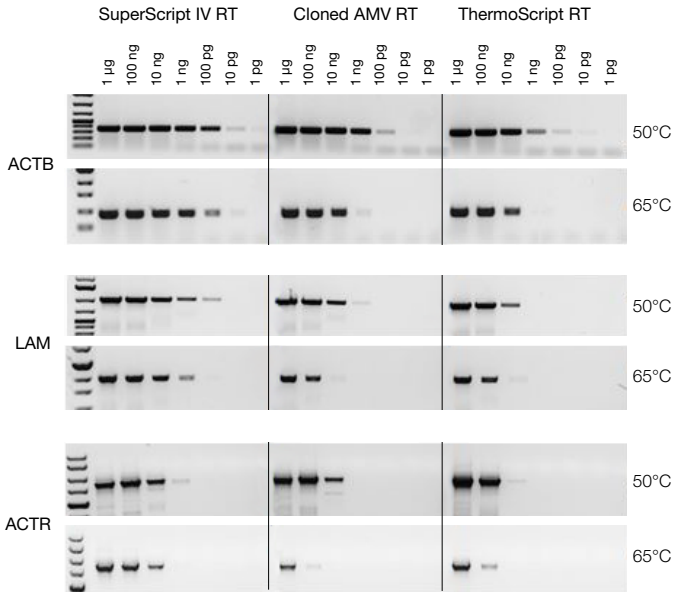

Figure 3. Sensitivity of SuperScript IV RT, AMV RT, and ThermoScript RT.

In each case, SuperScript IV RT showed the highest sensitivity, producing a detectable product at 10- to 100-fold lower input RNA than the other two RTs (Figure 3). At both the optimal temperature and the challenging 65°C temperature, SuperScript IV RT outperformed AMV RT and ThermoScript RT for all three targets. The ability to detect longer targets, such as ACTR, indicates that the reduced RNase H activity and improved thermostability of SuperScript IV RT allows it to synthesize more full-length cDNA.

Performance with degraded RNA samples

Researchers are working with increasingly difficult sample sources where RNA becomes degraded during the purification process. SuperScript IV RT, cloned AMV RT, and ThermoScript RT performance was evaluated using degraded total HeLa RNA (RIN 1) and human placenta RNA (RIN 2.6) at 50°C and 65°C. At 50°C, the reaction time was 10 minutes for SuperScript IV RT and 60 minutes for cloned AMV and ThermoScript RTs. At 65°C, the reaction time was 60 minutes for all three enzymes. cDNA was used for qPCR with TaqMan Assays for 18S rRNA, GAPDH, HPRT1, and TBP targets (Figure 4).

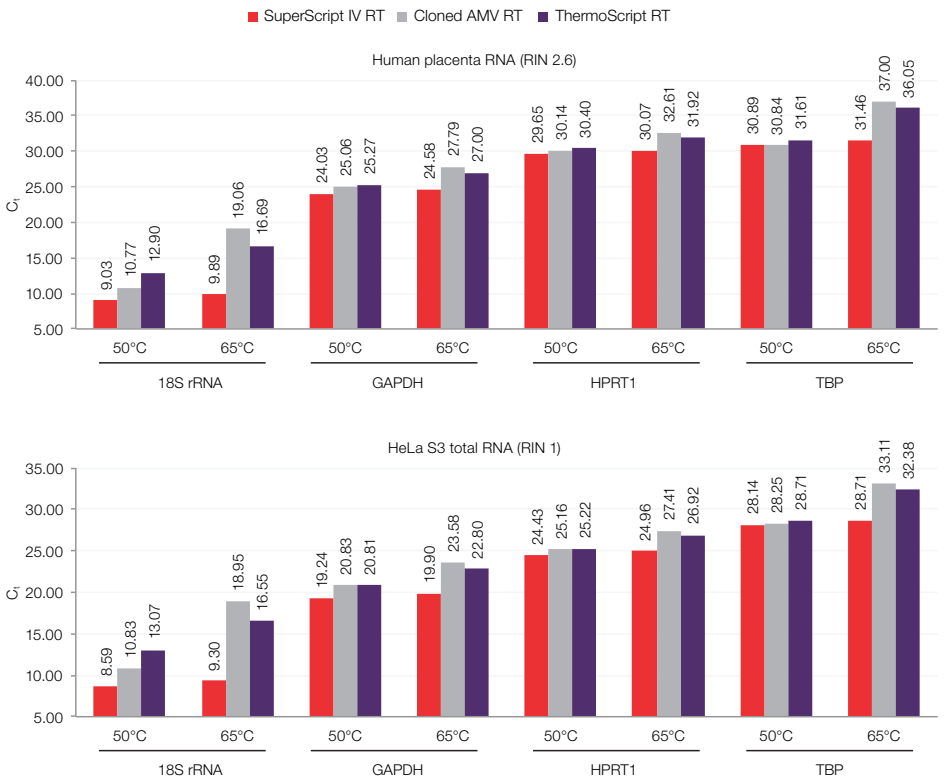

Figure 4. Performance of SuperScript IV RT, AMV RT, and ThermoScript RT with degraded RNA templates.

For all conditions tested, SuperScript IV RT produced the highest cDNA yields and the lowest C_t values. Additionally, the C_t values for SuperScript IV RT at 65°C were nearly identical to their values at the optimal 50°C ($\Delta C_t < 1$), whereas the performance of the other two products was substantially worse at the higher temperatures, with C_t values between 1.5 and 8 cycles higher than at the optimal temperature. These results demonstrate the superior sensitivity, thermostability, and overall performance of SuperScript IV RT with degraded RNA.

Conclusion

As research moves toward challenging samples and applications and demands on reverse transcriptases increase, enzyme performance must keep pace. While AMV-derived RTs used to have a thermostability advantage over wild type MMLV RT, the technology has evolved, and better MMLV RT–based enzymes are available. Thermo Fisher Scientific has engineered SuperScript IV RT, an MMLV RT–based enzyme with significantly improved processivity (50-fold higher than wild type MMLV RT, SuperScript II RT, and SuperScript III RT, and 3-fold higher than AMV RT and ThermoScript RT). SuperScript IV RT outperforms other RTs, especially under challenging reaction conditions at temperatures up to 65°C, in the presence of inhibitors, and with degraded RNA. In addition, reactions with SuperScript IV RT are faster and more sensitive. Researchers can confidently replace AMV RT or ThermoScript RT in their current protocols with SuperScript IV RT.

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References

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Ordering information

Product	Quantity	Cat. No.
SuperScript IV Reverse Transcriptase	2,000 units	18090010
	10,000 units	18090050
	4 x 10,000 units	18090200
SuperScript IV First-Strand Synthesis System	50 reactions	18091050
	200 reactions	18091200
SuperScript IV VILO Master Mix	50 reactions	11756050
	500 reactions	11756500
SuperScript IV VILO Master Mix with ezDNase Enzyme	50 reactions	11766050
	500 reactions	11766500

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