SuperScript IV Reverse Transcriptase

Abstract

Surveys and interviews conducted over a three-year period revealed that researchers are not satisfied with their current reverse transcriptases (RTs), and are performing reactions with increasingly difficult samples such as poorly purified RNA that contains inhibitors, RNA from plants, formalin-fixed, paraffin-embedded (FFPE) samples that are typically degraded, and unpurified RNA (direct reverse transcription). To meet this performance gap, we developed Invitrogen™ SuperScript™ IV RT. Based on internal experimental evidence, it is the most robust RT compared to other enzymes when used with difficult RNA samples. Characterization of SuperScript IV RT was performed in the context of imperfect situations where users do not have ideal RNA samples. Using a variety of stringent assays, this paper demonstrates that SuperScript IV RT possesses superior performance in the presence of a variety of inhibitors, such as alcohols, salts, detergents, heparin, hematin, bile salts, and formalin, typically found in sample preparation reagents, cell lines, blood, feces, and FFPE samples. In our experiments, SuperScript IV RT maintained the highest sensitivity with degraded RNA (RNA integrity number (RIN): 1–3) and unpurified RNA samples. Furthermore, this enzyme retains the lowest variability with different amounts of input RNA. Finally, SuperScript IV RT is found to be the most thermostable (100% activity up to 56°C and 90% activity at 60°C) and processive (up to 9 kb in just 10 min) RT. To demonstrate even greater value, the attributes of SuperScript IV RT mentioned above were benchmarked against other leading RTs.

Introduction

RTs are a class of enzymes that synthesize cDNA from an RNA template. Traditionally, scientists used RTs to clone genes and study expressed genes from whole organisms or cells. However, the scientific landscape in gene sequencing has drastically changed in recent times to include single-cell analysis, sample preparation–free analysis, and next-generation sequencing, requiring new RTs with single-copy sensitivity, speed, resilience to difficult samples, and thermoreactivity. To meet these demands, we have engineered a new RT. SuperScript IV RT is the newest member of the SuperScript™ family of RTs, which is known for its quality and reliability in cDNA synthesis. Functions of RTs are described by several attributes—processivity, sensitivity, reproducibility, and yield. While many RTs meet users' expectations in these attributes with high-quality RNA samples, in cases where inhibitors are present, RNAs are degraded, and sample preparation is difficult, RTs from other suppliers fall short of expectations while SuperScript IV RT excels.

Materials and methods

RNA purification

Wheat germ and flax seeds purchased from a grocery store were ground to a fine powder in liquid nitrogen. RNA was extracted and purified using the Invitrogen™ PureLink™ Plant RNA Reagent (Thermo Fisher Scientific, Cat. No. 12322012) and its accompanying protocol. Total RNA was quantitated using the Thermo Scientific™ NanoDrop™ instrument and treated with DNase I (Thermo Fisher Scientific, Cat. No. 18068015). RNA quality was assessed using the Bio-Rad Experion™ Automated Electrophoresis System and agarose gel electrophoresis with ethidium bromide staining.

Degraded RNA preparation

HeLa RNA (Thermo Fisher Scientific, Cat. No. AM7852) and *Arabidopsis* RNA (BioChain, Cat. No. R1634310) were degraded to RIN 1–3 by addition of $MgCl_2$ to a final concentration of 1 mM and heated to 95°C for 15 min.

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Unpurified RNA (direct reverse transcription) samples

Pelleted HeLa cells, *Arabidopsis* tissue, wheat germ tissue, and flax seed tissue were ground to a fine powder in liquid nitrogen. The powder was transferred to a microfuge tube and Tris-EDTA (TE) was added, vortexed, and centrifuged to pellet debris. Resulting clarified supernatant was transferred into a fresh microfuge tube. Prior to reverse transcription, EDTA and DTT were added to the supernatant to a final concentration of 1 mM and 5 mM, respectively, and heated to 95°C for 10 min.

Commercial RNA samples

Commercial RNA used included Invitrogen™ Cervical Adenocarcinoma (HeLa-S3) Total RNA (Thermo Fisher Scientific, Cat. No. AM7852), *Arabidopsis* Total RNA (BioChain, Cat. No. R1634310), Rat Brain Total RNA (Clontech, Cat. No. 636653), 0.5–10 kb RNA Ladder (Thermo Fisher Scientific, Cat. No. 15623200), and Invitrogen™ Millennium™ RNA Markers (Thermo Fisher Scientific, Cat. No. AM7150).

SuperScript IV cDNA synthesis protocol

The example procedure below (Schematic 1) shows appropriate volumes for a single, 20 µL reverse transcription reaction. For multiple reactions, prepare a master mix of components common to all reactions to minimize pipetting error, then dispense appropriate volumes into each reaction tube prior to adding annealed template RNA and primers.

qPCR

The reverse transcription reaction contributed up to 10% of the total qPCR reaction volume. Applied Biosystems™ TaqMan® Assays for the gene targets are indicated in the figures. EXPRESS qPCR SuperMix (Thermo Fisher Scientific, Cat. No. 1178501K) and the Applied Biosystems™ ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Cat. No. 4453536) were used for qPCR. C_t values were normalized to SuperScript IV RT using the equation: Normalized Y values = 2^(C_t SuperScript IV – C_t competitor).

Endpoint PCR

The reverse transcription reaction contributed up to 10% of the total PCR reaction volume. Invitrogen™ Platinum™ *Taq* DNA Polymerase High Fidelity and its accompanying protocol were used. 10 µL of PCR reaction was resolved using agarose gel electrophoresis and visualized by ethidium bromide staining.

Activity assay for thermostability

Reverse transcriptases were preincubated at the indicated temperatures for an indicated amount of time in 1X reaction buffer and 100 ng/µL Invitrogen™ UltraPure™ Calf Thymus DNA Solution (Thermo Fisher Scientific, Cat. No. 15633019). Following preincubation, enzymes were added to a 1X polymerization mix containing 1X reaction buffer, 2 mM oligo(dT)$_{16}$, 0.02 µg poly(rA) (GE Healthcare, Cat. No. 45-001-356), 2 mM dTTP, and 1X EvaGreen™ dye (Biotium, Cat. No. 31000). Extension was performed for 10 min at room temperature. Fluorescence reading was recorded using the SpectraMAX™ Gemini EM plate reader (Molecular Devices) with an excitation of 490 nm and emission of 520 nm. Percent activity remaining after heat treatment was determined by normalizing to the fluorescence reading without heat treatment that is set to 100%.

Steps	Procedure	Procedure details
1	**Anneal primer to template RNA**	a. Combine the following components in a reaction tube. **Note**: Consider the volumes for all components listed in steps 1 and 2 to determine the correct amount of water required to reach your final reaction volume. Component / Volume: 50 µM oligo (dT)$_{20}$ primer, 50 µM random hexamers, or 2 µM gene-specific reverse primer — 1 µL 10 mM dNTP mix (10 mM each) — 1 µL Template RNA (10 pg–5 µg total RNA or 10 pg–500 ng mRNA) — up to 11 µL DEPC-treated or nuclease-free water — to 13 µL b. Mix and briefly centrifuge the components. c. Heat the RNA-primer mix at 65°C for 5 min., and then incubate on ice for at least 1 min.
2	**Prepare RT reaction mix**	a. Vortex and briefly centrifuge the 5X SuperScript IV RT buffer. b. Combine the following components in a reaction tube. Component / Volume: 5X SuperScript IV RT buffer — 4 µL 100 mM DTT — 1 µL RNaseOUT Recombinant RNase Inhibitor — 1 µL SuperScript IV Reverse transcriptase (200 U/µL) — 1 µL c. Cap the tube, mix, and then briefly centrifuge the contents.
3	**Combine annealed RNA and RT reaction mix**	Add RT reaction mix to the annealed RNA.
4	**Incubate reactions**	a. If using random hexamer, incubate the combined reaction mixture at 23°C for 10 min., and then proceed to step b. If using oligo (dT)$_{20}$ or gene-specific primer, procced directly to step b. b. Incubate the combined reaction mixture at 50–55°C for 10 min. c. Inactivate the reaction by incubating it at 80°C for 10 min.
5	**Optional: remove RNA**	**Note**: Amplification of some PCR targets (>1 kb) may require removal of RNA. To remove RNA, add 1 µg *E. coli* RNase H, and incubate at 37°C for 20 min.
6	**PCR amplification**	Use your RT reaction immediately for PCR amplification or store it at –20°C. **Note**: As a recommended starting point for PCR, reverse transcription reaction (cDNA) should compose 10% of the total reaction volume.

Schematic 1. Steps for first-strand cDNA synthesis using Invitrogen™ SuperScript™ IV First-Strand cDNA Synthesis System.

First-strand cDNA synthesis for thermostability

Reverse transcription was performed as described above using oligo(dT)$_{20}$ and 500 ng Millennium RNA Markers, with the exception of reaction temperature that ranged from 50 to 65°C. Invitrogen™ SuperScript™ III RT reactions were performed according to the accompanying protocol with the exception of reaction temperature that ranged from 50 to 65°C with a reaction time of 50 min. First-strand cDNA was resolved by alkaline gel electrophoresis and cDNA was stained using Invitrogen™ SYBR™ Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Cat. No. S11494). Sodium hydroxide hydrolyzed all RNA, resulting in only visualization of cDNA. Each cDNA band was measured using the TotalLab™ image analysis module and volumes were summed up for each reaction temperature. Percent activity was calculated from the ratio of total volume at each reaction temperature to the total volume at 50°C.

Results

RT-qPCR performance using SuperScript IV RT and RTs from other suppliers in the presence of inhibitors

Trace amounts of reagents used during RNA isolation can cause problems during reverse transcription. For example, some reagents used to lyse cells, such as SoluLyse™ and BugBuster™ reagents, contain detergents. TRIzol™ reagent, used to extract RNA from cells and tissues, contains phenol. Salts such as guanidinium chloride, guanidinium isothiocyanate, ammonium acetate, and lithium chloride are used in multiple steps during RNA isolation and precipitation. FFPE samples may still contain formalin and paraffin. Inhibitors may also be inherent in the biological sample source such as hematin, a drug found in blood, bile salt, found in blood and feces, and humic acid, found in soil and thus, on plants. To test how chemical compounds affect reverse transcription efficiency, various inhibitors were added to total HeLa RNA prior to the oligo(dT)$_{20}$ annealing step. Concentrations indicated in Figure 1 are the final concentrations of inhibitors in complete reverse transcription reactions. Reverse transcription with SuperScript IV RT, SuperScript III RT, and six other RTs (P, T, BR, Q, BL, and N) from other suppliers, followed by qPCR, revealed that SuperScript IV RT had the most consistent results with all of the inhibitors tested (Figure 1).

SuperScript IV RT functions exceedingly well, better than all other tested enzymes in the presence of ethanol, SoluLyse reagent, salts, hematin, and humic acid. The enzyme from supplier N gave ~5-fold more product than SuperScript IV RT. The reactivity of SuperScript IV RT is almost equivalent to that of supplier BL enzyme in BugBuster reagent. Supplier BR and BL enzymes functioned better than SuperScript IV in formalin. Since the majority of reverse transcriptases functioned more uniformly in paraffin, the wax component in FFPE samples caused little to no inhibition in reverse transcription. In summary, SuperScript IV RT demonstrated the most consistent performance in the presence of a variety of inhibitors.

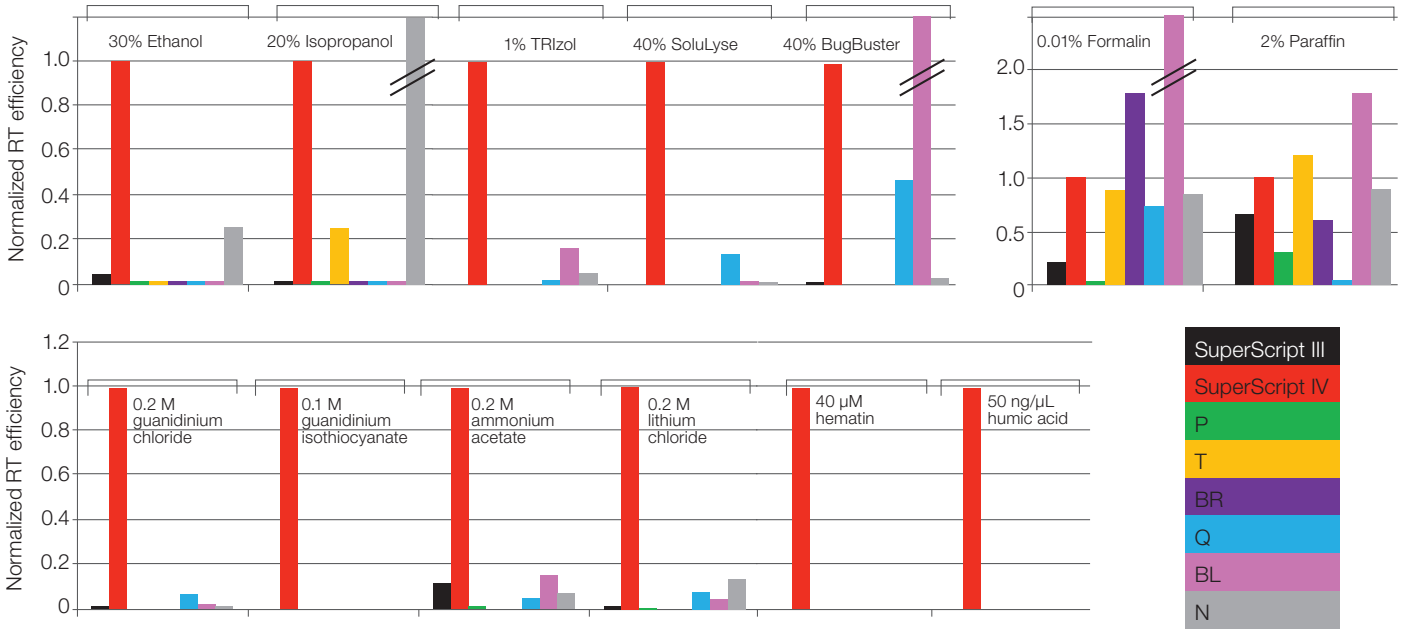

Figure 1. RT-qPCR performance with SuperScript IV RT and RTs from other suppliers in the presence of inhibitors.

First-strand cDNA synthesis performance using SuperScript IV RT and RTs from other suppliers in the presence of inhibitors

In addition to studying the effect of inhibitors on RT-qPCR, direct analysis of RT activity in the presence of inhibitors was also performed. RT-qPCR may hide the actual performance of RTs because targets are usually less than 200 bp and thus, much easier to reverse-transcribe. Therefore, analysis of first-strand cDNA synthesis using RNA targets of different sizes gives a more accurate representation of RT performance. First-strand cDNAs resulting from reverse transcription of a 0.5–10 kb RNA ladder were resolved by alkaline gel electrophoresis that degrades RNA. Single-stranded cDNA was visualized by staining with SYBR Gold Nucleic Acid Gel Stain. Contrary to the results observed with RT-qPCR of small targets, the reverse transcription of longer targets revealed that the enzymes are more susceptible to inhibitors in general (Figure 2). In the presence of isopropanol, the enzyme from supplier N does not make any cDNAs of size 0.5 kb or below, while SuperScript IV RT can still synthesize up to 1 kb. In BugBuster reagent, SuperScript IV RT can synthesize up to 8 kb, while supplier BL enzyme makes very little cDNA and stops after ~1.5 kb. The same effect was observed for formalin, where supplier BL and BR enzymes had trouble with cDNA yield and length, while SuperScript IV RT retained most of its activity. Other inhibitors tested by direct cDNA analysis encompassed inhibitors inherent in biological samples such as heparin found in animal blood, tissue, and cells and bile salts found in blood and feces. Using analysis of just first-strand cDNA, SuperScript IV RT exhibited superior performance compared to reverse transcriptases from all other suppliers. The exception was with bile salts, where SuperScript III RT also retained most of its activity. Positive control experiments where no inhibitors were added to reverse transcription revealed that BL, P, Q, and BR enzymes from other suppliers cannot synthesize targets greater than 2 kb even when conditions are ideal.

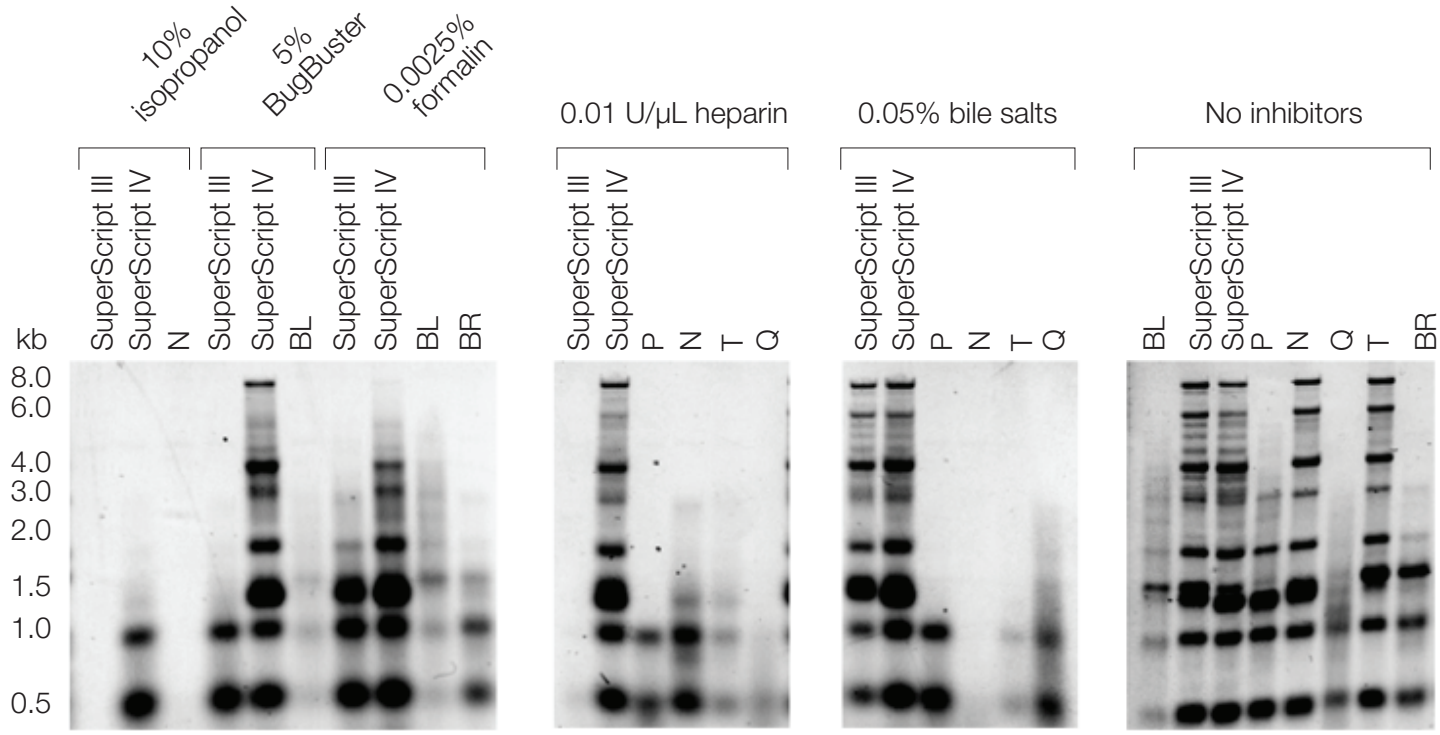

Figure 2. First-strand cDNA synthesis performance using SuperScript IV RT and RTs from other suppliers.

Performance of SuperScript IV RT and RTs from other suppliers with degraded RNA samples

Sensitivity, or the ability of RTs to generate cDNA from very little input RNA, is an important attribute to this class of enzyme. Furthermore, researchers are working with increasingly difficult sample sources where RNA becomes degraded during the RNA purification process, resulting in even lower yields of full-length transcripts. RT sensitivity was therefore evaluated in the context of degraded RNA from HeLa cells, *Arabidopsis* tissue, wheat germ tissue, and flax seed tissue (Figure 3). RIN values ranged between 1 and 3, in contrast to high-quality, intact RNA with an RIN greater than 8. Nine targets were evaluated by qPCR and in every case, SuperScript IV RT was more sensitive than all other enzymes tested.

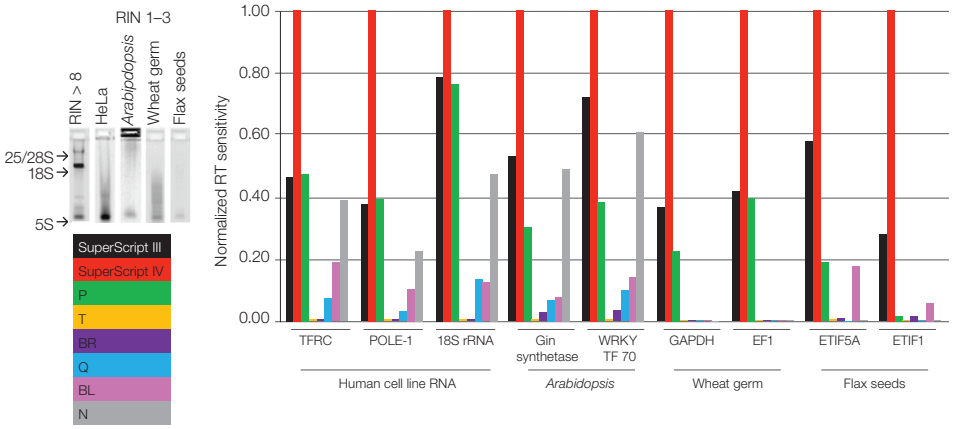

Figure 3. Performance of SuperScript IV RT and RTs from other suppliers with degraded RNA samples.

Performance of SuperScript IV RT and RTs from other suppliers with unpurified RNA samples

The data presented earlier demonstrates that SuperScript IV RT is the most robust and sensitive RT in the presence of inhibitors and degraded RNA. However, researchers face a combination of problems with their samples. To challenge RTs with a "real world" scenario, direct reverse transcription was performed with unpurified RNA samples. Ground 293 cells, *Arabidopsis* tissue, and wheat germ tissue were mixed with TE to dissolve RNA, which was then added directly to reverse transcription reactions. Thus, the input samples have very low copies of transcripts and a variety of inhibitors. For seven qPCR targets in 293 cells, *Arabidopsis* tissue, and wheat germ tissue, SuperScript IV RT is the most sensitive (Figure 4). Most RTs performed well for GAPDH in wheat germ. Nonetheless, SuperScript IV RT is the most consistently robust enzyme for direct reverse transcription.

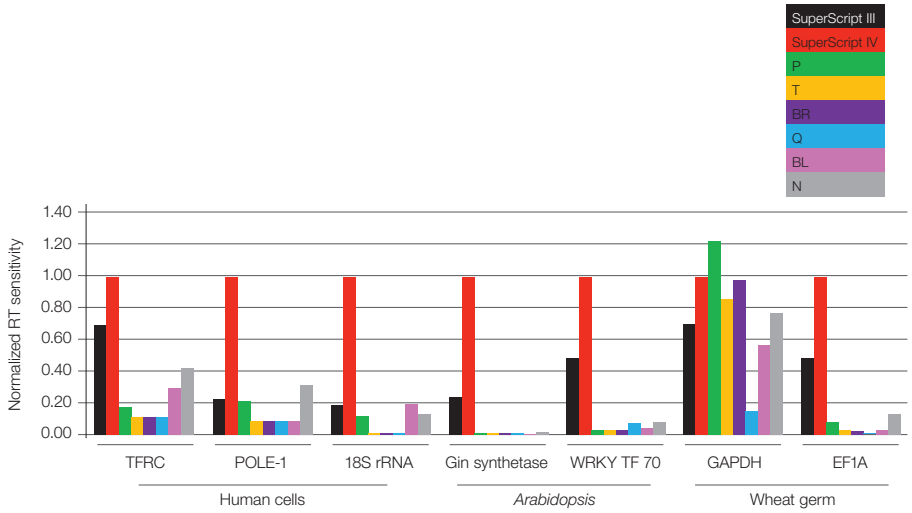

Figure 4. Performance of SuperScript IV RT and RTs from other suppliers with unpurified RNA samples.

Sensitivity and variability of SuperScript IV RT and RTs from other suppliers

A more thorough investigation of RT sensitivity and variability was performed in the context of degraded RNA purified from *Arabidopsis* (RIN: 1–3). 1, 10, and 100 ng of degraded total RNA was used in reverse transcription reactions. Triplicate reverse transcription reactions were performed for each input RNA amount. Triplicate qPCR reactions for two *Arabidopsis* targets were performed for each reverse transcription reaction. For both targets, SuperScript IV RT resulted in the lowest C_t values over all input RNA amounts (Figure 5). A plot of standard deviations for all input RNA amounts revealed that not only did SuperScript IV RT have the lowest standard deviation, but also resulted in the least amount of variation over all three input RNA amounts, compared to other RTs.

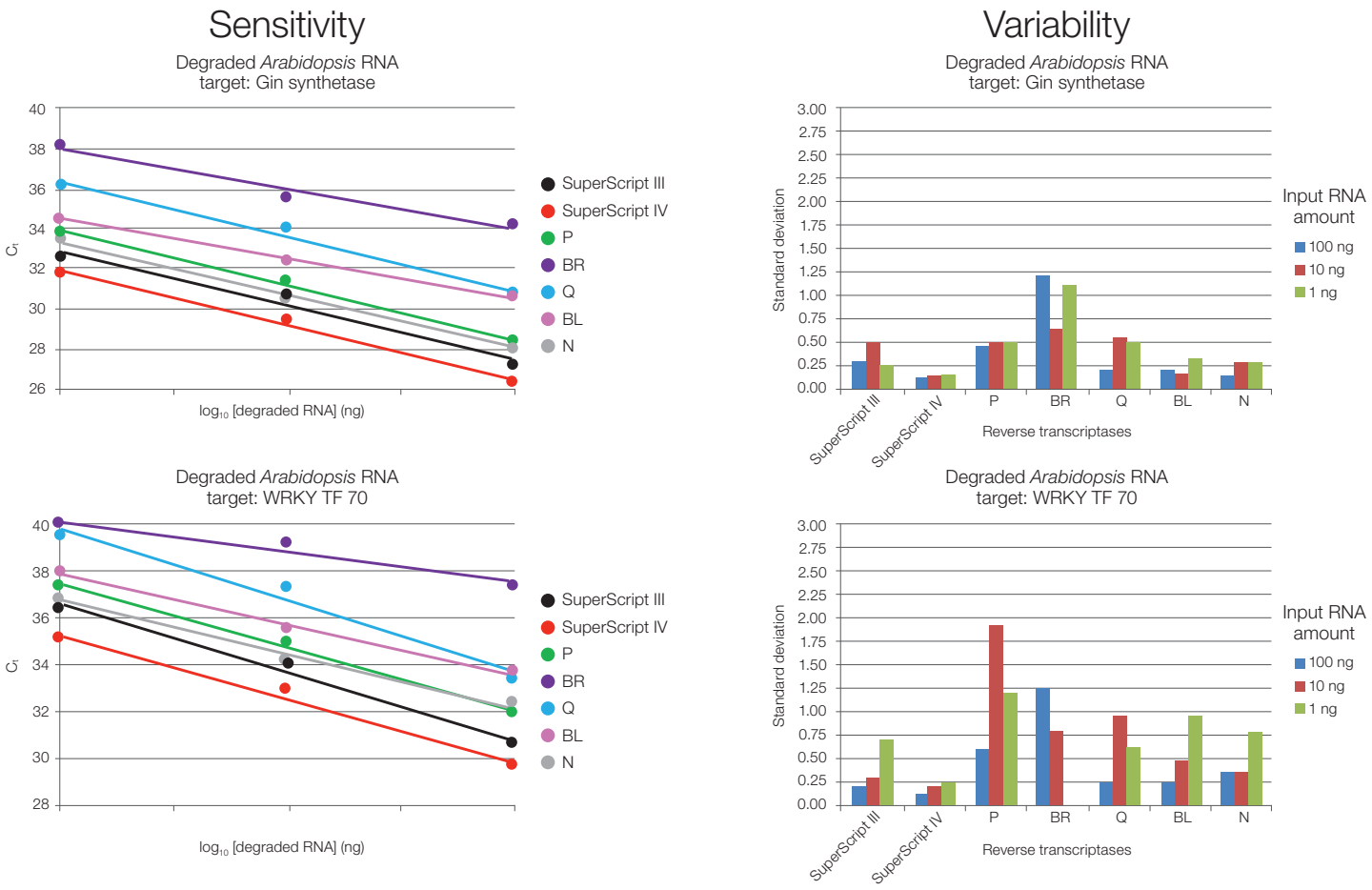

Figure 5. Sensitivity and variability of SuperScript IV RT and RTs from other suppliers with degraded plant RNA.

Processivity study of SuperScript IV RT and RTs from other suppliers

Processivity is the ability of a polymerase to perform consecutive nucleotide additions without releasing the RNA template. The more processive an enzyme, the longer the cDNA that can be synthesized and the faster the enzyme is in making full-length cDNA. The RT speed and produced cDNA length were assessed using a 0.5–9 kb RNA ladder. RT reaction time was 10 min. Only SuperScript IV RT synthesized up to 9 kb cDNA (Figure 6). SuperScript III RT can synthesize up to 5 kb, while the rest of the enzymes failed to synthesize cDNA up to 3 kb, indicating that SuperScript IV RT is the most processive RT.

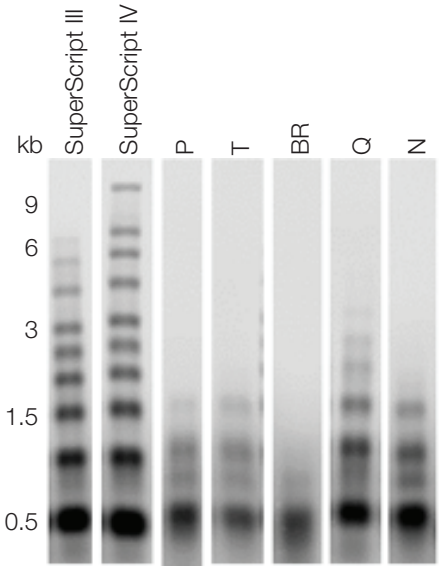

Figure 6. Processivity study of SuperScript IV RT and RTs from other suppliers.

Thermostability study of SuperScript IV RT and RTs from other suppliers

Thermostability of the RTs was evaluated by preincubating at 50°C from 0 to 90 min. Following preincubation, polymerization activity was measured using a fluorescence activity assay. Only SuperScript III and IV RTs remained active at 50°C for a sustained period of time (Figure 7). The thermostability of SuperScript III and IV RTs was more stringently evaluated from 50°C to 65°C by measuring first-strand cDNA synthesis of a 0.5–9 kb RNA ladder. The cDNA yield and length dropped drastically with temperatures slightly above 50°C for SuperScript III RT. However, SuperScript IV RT sustained 100% activity up to 56.4°C and 70% activity up to 65°C. The ability of SuperScript IV RT to function at higher temperatures enables the reverse transcription of RNA targets with strong secondary structure.

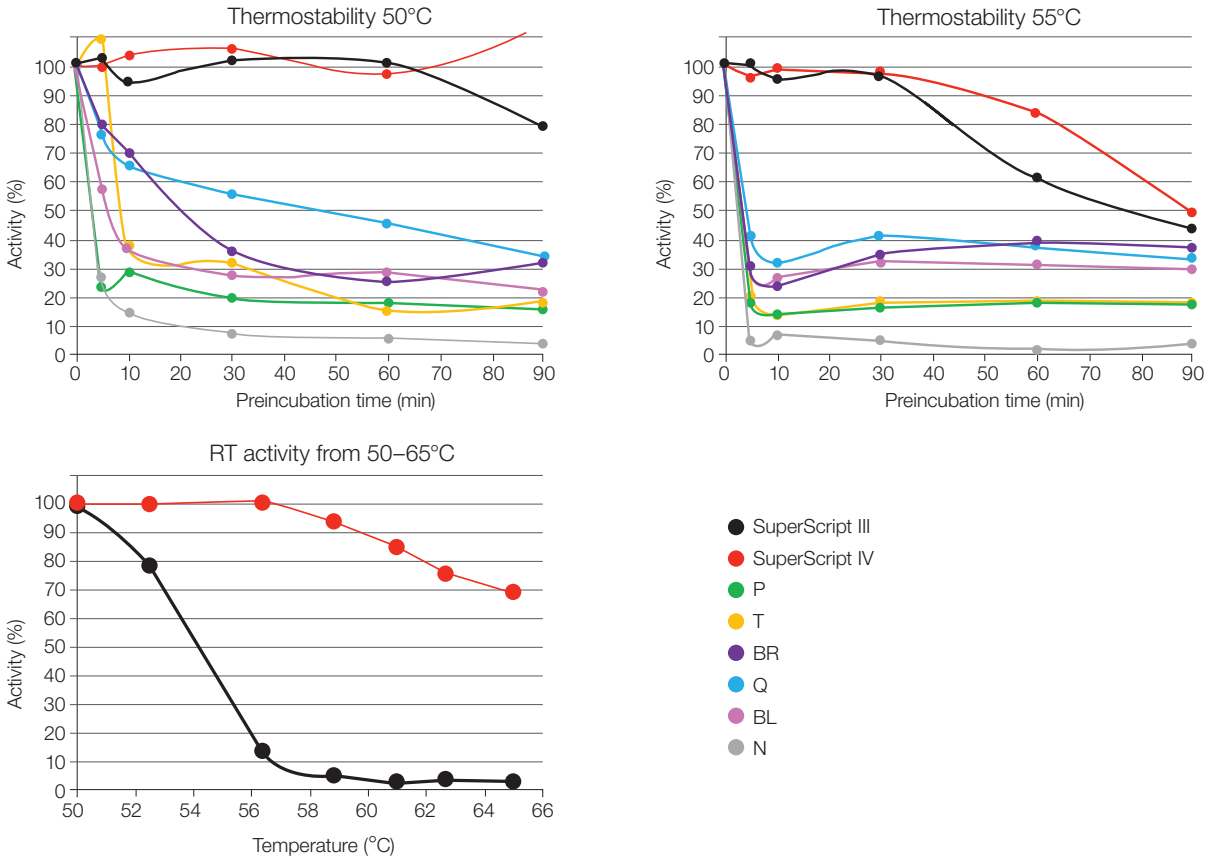

Figure 7. Thermostability study of SuperScript IV RT and RTs from other suppliers.

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cDNA length study with SuperScript IV RT and RTs from other suppliers

Although most processed RNAs are around 3 kb, there are genes that exceed this size. To evaluate the performance of RTs with longer transcripts, reverse transcription followed by endpoint PCR was performed for a 12.3 kb *in vitro* transcript. The reverse transcription protocol was modified to include a gene-specific RT primer. Reverse transcription reaction incubation was 30 min at recommended reaction temperatures, 15 min at 5°C above the recommended reaction temperature, and 15 min at 10°C above the recommended reaction temperature for all enzymes tested to accommodate for secondary structure and large transcript size. Subsequent PCR resulted in a 12.3 kb product only in the SuperScript IV RT reaction, while all other RTs produced smears and smaller products (Figure 8).

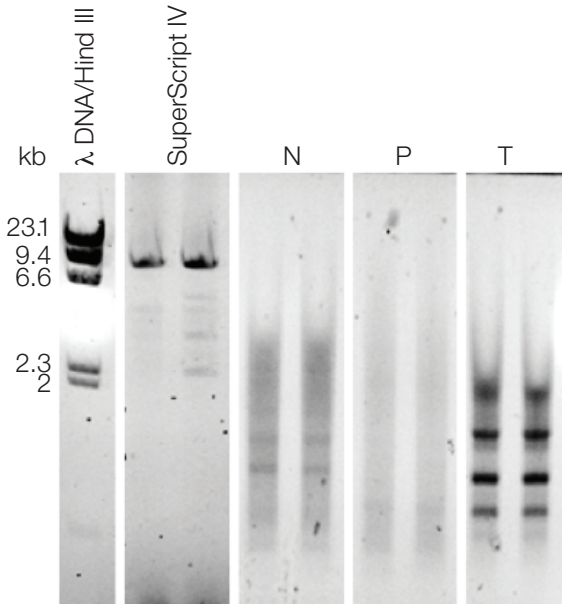

Figure 8. cDNA length study using SuperScript IV RT and RTs from other suppliers.

Conclusion

Scientific advancement requires researchers to continually undertake new challenges and obtain results more rapidly. We strive to meet the needs of the scientific community by continuously working on improving reagents and tools for current and future research. SuperScript IV RT is such an example. SuperScript IV RT enables scientists to quickly progress their research by providing sensitive and reliable RNA analysis from difficult sample sources that contain low copies of target RNA, degraded RNA, and inhibitors. SuperScript IV RT raises the bar by enabling scientists to analyze RNA without purification.

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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