SuperScript IV Reverse Transcriptase

Abstract

Survey and interview studies conducted over a three-year period revealed that researchers are not satisfied with their current reverse transcriptase and are performing reactions with increasingly difficult samples, such as poorly purified RNA that contains inhibitors, RNA from plants, FFPE samples that are typically degraded, and unpurified RNA (direct reverse transcription). To meet this performance gap we produced SuperScript™ IV Reverse Transcriptase (RT), and internal experimental evidence shows that it is the most robust reverse transcriptase compared to other enzymes when used with difficult RNA samples. SuperScript IV RT characterization 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 most sensitivity with degraded RNA (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 the most thermostable (100% activity up to 56°C and 90% activity at 60°C) and processive (up to 9 kb in just 10 minutes) reverse transcriptase. To demonstrate even greater value, the SuperScript IV RT attributes mentioned above were benchmarked against other leading reverse transcriptases.

Introduction

Reverse transcriptases are a class of enzymes that synthesizes cDNA from a RNA template. Traditionally, scientists used reverse transcriptases to clone genes and study expressed genes from whole organisms or cells. However, the scientific landscape has drastically changed to include single-cell analysis, sample preparation–free analysis, and next-generation sequencing, requiring new reverse transcriptases with single-copy sensitivity, speed, resilience to difficult samples, and thermoreactivity. To meet these demands, we have engineered a new reverse transcriptase. SuperScript IV RT is the newest member of the SuperScript family of reverse transcriptases, which is known for its quality and reliability in cDNA synthesis. Reverse transcriptase functions are described by several attributes—processivity, sensitivity, reproducibility, and yield. While many reverse transcriptases 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, competitor reverse transcriptases fall short of expectations while SuperScript IV RT excels.

Materials and methods

RNA purification

Wheat germ and flax seeds purchased from the grocery store were ground to a fine powder in liquid nitrogen. RNA was extracted and purified using PureLink™ Plant RNA Reagent (Thermo Fisher Scientific, Cat. No. 12322012) and the accompanying protocol. Total RNA was quantitated by 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) was degraded to RIN 1–3 by addition of $MgCl_2$ to a final concentration of 1 mM and heated to 95°C for 15 minutes.

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 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 was added to the supernatant to a final concentration of 1 mM and 5 mM, respectively, and heated to 95°C for 10 minutes.

Reverse transcription

Commercial RNA utilized included 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 RNA Millennium™ Markers (Thermo Fisher Scientific, Cat. No. AM7150).

qPCR

Reverse transcription reaction contributed up to 10% of the total qPCR reaction volume. TaqMan™ Assays for the gene targets are indicated in the figures. EXPRESS qPCR SuperMix (Thermo Fisher Scientific, Cat. No. 1178501K) and the ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific, Cat. No. 4453536) were utilized. C_t values were normalized to SuperScript IV RT using the equation: Normalized Y values = $[2^{(Ct_{SSIV} - Ct_{SSIV})}]/[2^{(Ct_{competitor} - Ct_{SSIV})}]$

Endpoint PCR

Reverse transcription reaction contributed up to 10% of the total PCR reaction volume and utilized Platinum *Taq* DNA Polymerase High Fidelity and accompanying protocol. 10 µL of PCR reaction was resolved using agarose gel electrophoresis and visualized by ethidium bromide staining.

SuperScript® IV First-Strand cDNA Synthesis Reaction

The example procedure below shows appropriate volumes for a single 20-µL reverse transcription reaction. For multiple reaction, prepare a mastermix 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.

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: 50 µM Oligo $d(T)_{20}$ primer, 50 µM random hexamers, or 2 µM gene-specific reverse primer — Volume: 1 µL Component: 10 mM dNTP mix (10 mM each) — Volume: 1 µL Component: Template RNA (10 pg–5 µg total RNA or 10 pg–500 ng mRNA) — Volume: up to 11 µL Component: DEPC-treated or nuclease-free water — Volume: to 13 µL b. Mix and briefly centrifuge the components. c. Heat the RNA-primer mix at 65°C for 5 minutes, and then incubate on ice for at least 1 minute.
2 	**Prepare RT reaction mix**	a. Vortex and briefly centrifuge the 5x SSIV Buffer. b. Combine the following components in a reaction tube. Component: 5× SSIV Buffer — Volume: 4 µL Component: 100 mM DTT — Volume: 1 µL Component: RNaseOUT™ Recombinant RNase Inhibitor — Volume: 1 µL Component: SuperScript® IV Reverse transcriptase (200 U/µL) — Volume: 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 minutes, and then proceed to step b. If using oligo $d(T)_{20}$ or gene-specific primer, directly procced to step b. b. Incubate the combined reaction mixture 50–55°C for 10 minutes. c. Inactivate the reaction by incubating it at 80°C for 10 minutes.
5 	***Optional*: Remove RNA**	**Note:** Amplification of some PCR targets (>1 kb) may require removal of RNA. To remove RNA, add 1 µ *E. coli* RNase H, and incubate 37°C for 20 minutes.
6 	**PCR amplification**	Use your TR 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.

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 Calf Thymus DNA (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 minutes at room temperature. Fluorescence was read using the Molecular Devices SpectraMAX™ Gemini EM plate reader 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%.

First-strand cDNA synthesis for thermostability

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

Results

SuperScript IV RT and competitor reverse transcriptase RT-qPCR performance in the presence of inhibitors

Trace amounts of reagents used during RNA isolation will cause problems with 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 tissue, contains phenol. Salts, such as guanidinium chloride, guanidinium isothiocyante, 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, possible inhibitors were added to total HeLa RNA prior to the oligo(dT)$_{20}$ annealing step. Concentrations indicated in the figures are the final concentrations of inhibitors in complete reverse transcription reactions. Reverse transcription with SuperScript IV RT (red bars), SuperScript III RT, and six other competitor reverse transcriptases (P, T, BR, Q, BL, and N) followed by qPCR revealed that SuperScript IV RT had the most consistent results with all the inhibitors

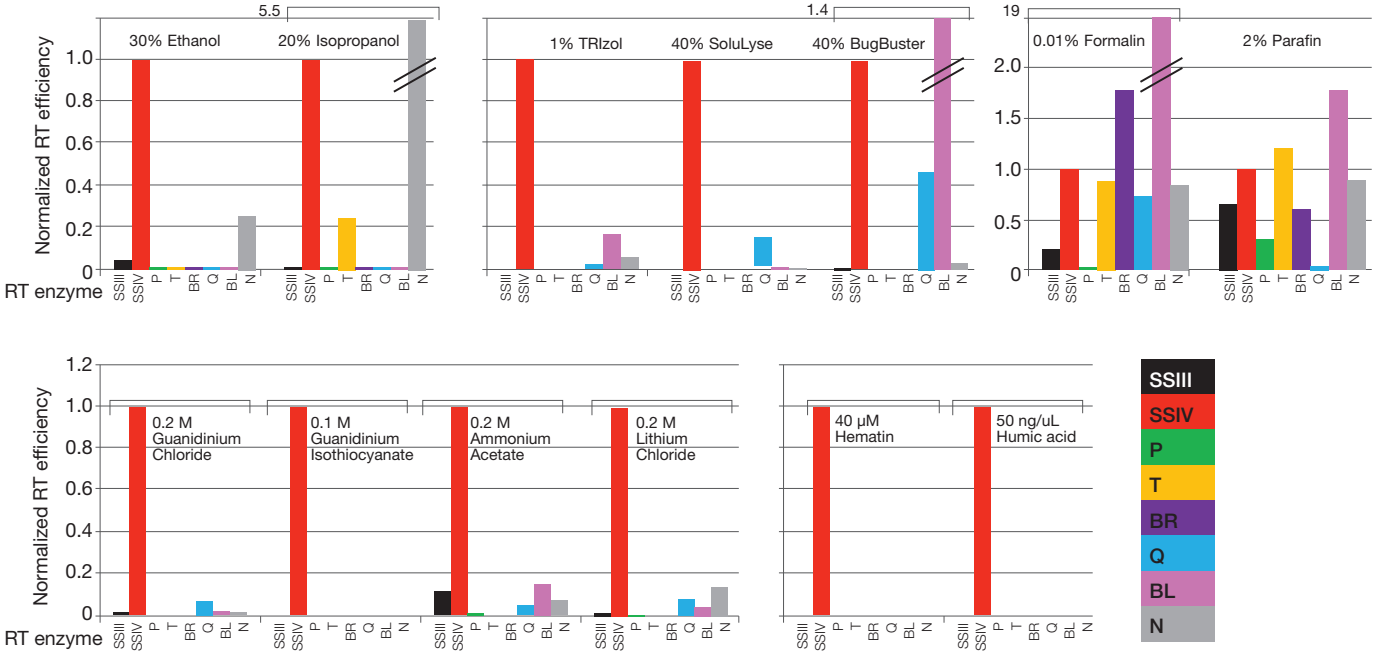

Figure 1. SuperScript IV RT and competitor reverse transcriptase RT-qPCR performance in the presence of inhibitors.

tested (Figure 1). SuperScript IV RT functions exceedingly better than all tested enzymes in the presence of ethanol, SoluLyse reagent, salts, hematin, and humic acid. Competitor N enzyme gave ~5-fold more product than SuperScript IV RT. Competitor BL enzyme is about equivalent to SuperScript IV RT in BugBuster reagent. Competitor BR and BL enzymes functioned better than SuperScript IV in formalin. Since the majority of reverse transcriptases functioned more comparably in paraffin, this wax component in FFPE samples caused little or no inhibition to reverse transcription. In summary, SuperScript IV RT demonstrated the most consistent performance in a variety of inhibitors.

SuperScript IV RT and competitor RT first-strand cDNA synthesis performance in the presence of inhibitors

In addition to studying the effect of inhibitors on RT-qPCR, direct analysis of reverse transcriptase 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 reverse transcriptase 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 isopropanol, Competitor N enzyme does not make any cDNAs of at least 0.5 kb, while SuperScript IV RT can still synthesize up to 1 kb. In BugBuster reagent, SuperScript IV RT can synthesize up to 8 kb, while Competitor BL enzyme makes very little cDNA and stops after ~1.5 kb. The same effect is observed for formalin where Competitor BL and BR enzymes have trouble with cDNA yield and length while SuperScript IV RT retains most of its activity. Other inhibitors tested by direct cDNA analysis encompass 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 exceeds all competitor reverse transcriptases. The exception is bile salts where SuperScript III RT also retains most of its activity. Positive control experiments where no inhibitors are added to reverse transcription revealed that Competitor BL, P, Q, and BR enzymes cannot synthesize targets greater than 2 kb even when conditions are ideal.

SuperScript IV RT and competitor RT performance with degraded RNA samples

Sensitivity, or the ability of reverse transcriptases 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. Reverse transcriptase sensitivity was therefore evaluated in the context of degraded RNA

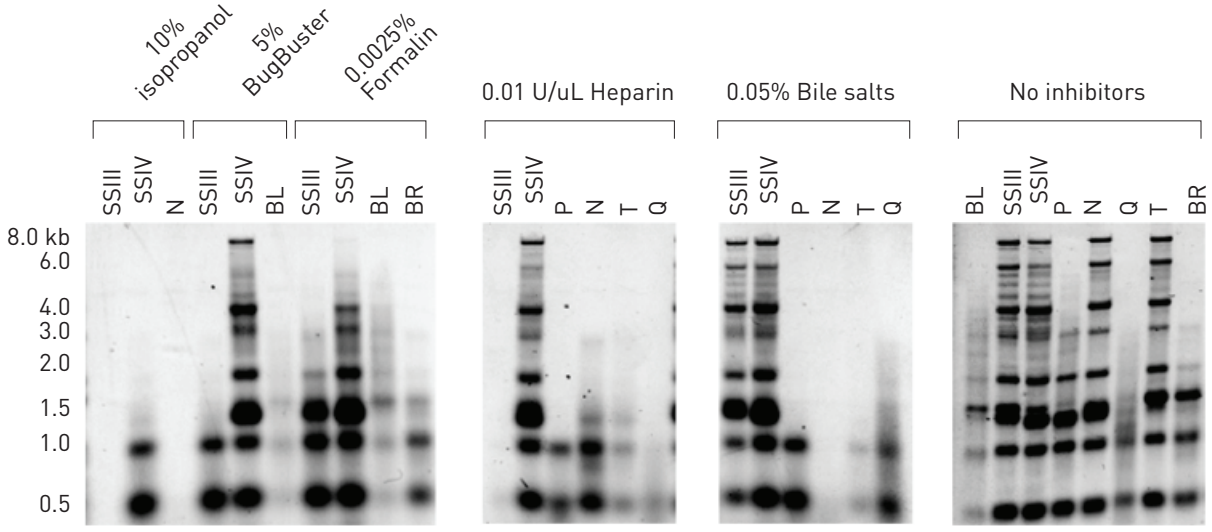

Figure 2. SuperScript IV RT and competitor RT first-strand cDNA synthesis performance in the presence of inhibitors.

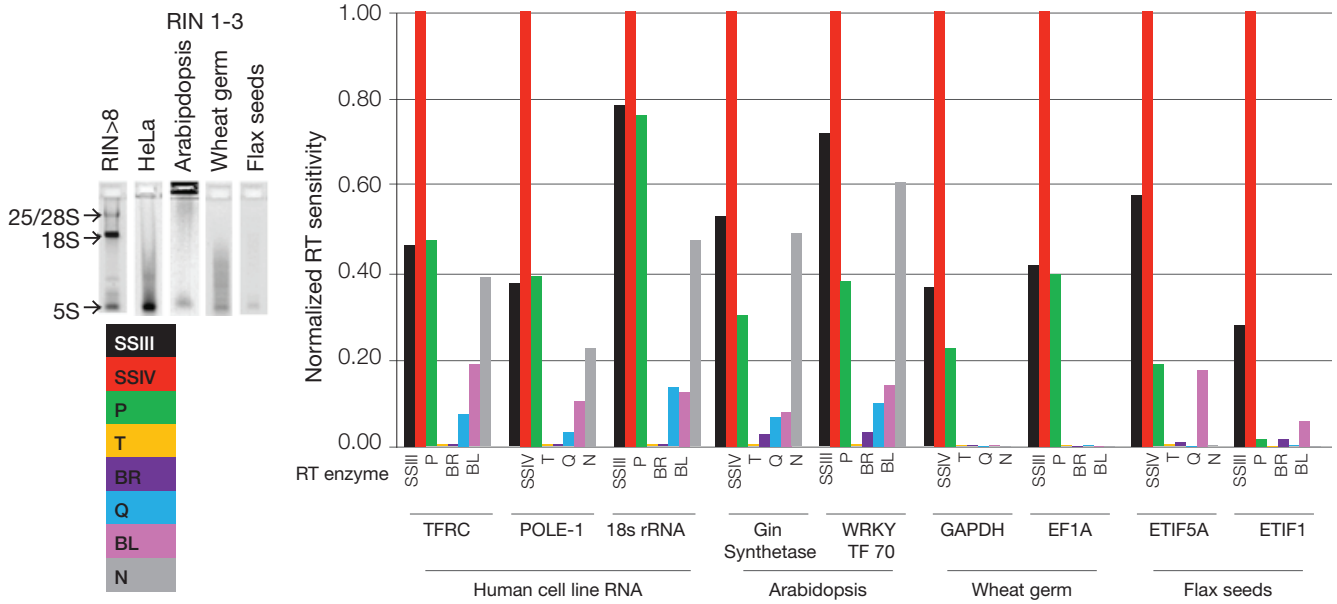

Figure 3. SuperScript IV RT and competitor RT performance with degraded RNA samples.

from HeLa cells, *Arabidopsis*, wheat germ, and flax seeds (Figure 3). RIN values ranged between 1 and 3, in contrast to high-quality intact RNA with a RIN greater than 8. Nine targets were evaluated by qPCR and in every case, SuperScript IV RT (red bars) was more sensitive than other enzymes tested.

SuperScript IV RT and competitor RT performance with unpurified RNA samples

The data presented earlier demonstrates SuperScript IV RT is the most robust and sensitive reverse transcriptase in the context of inhibitors and degraded RNA. However, researchers face a combination of problems with their samples. To challenge reverse transcriptases with a "real world" scenario, direct reverse transcription was performed with unpurified RNA samples. Ground 293 cells, *Arabipdopsis* tissue, and wheat germ tissue was 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 (red bars) is the most sensitive (Figure 4). Most reverse transcriptases performed well for GAPDH in wheat germ. Nonetheless, SuperScript IV RT is the most consistently robust enzyme for direct reverse transcription.

SuperScript IV RT and competitor RT sensitivity and variability with degraded plant RNA samples

A more thorough investigation of reverse transcriptase 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

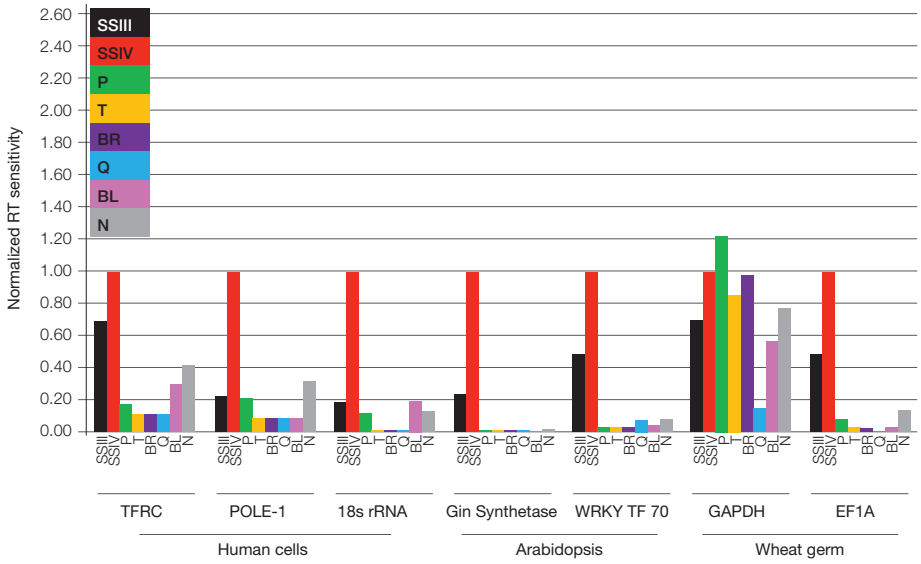

Figure 4. SuperScript IV RT and competitor RT performance with unpurified RNA samples.

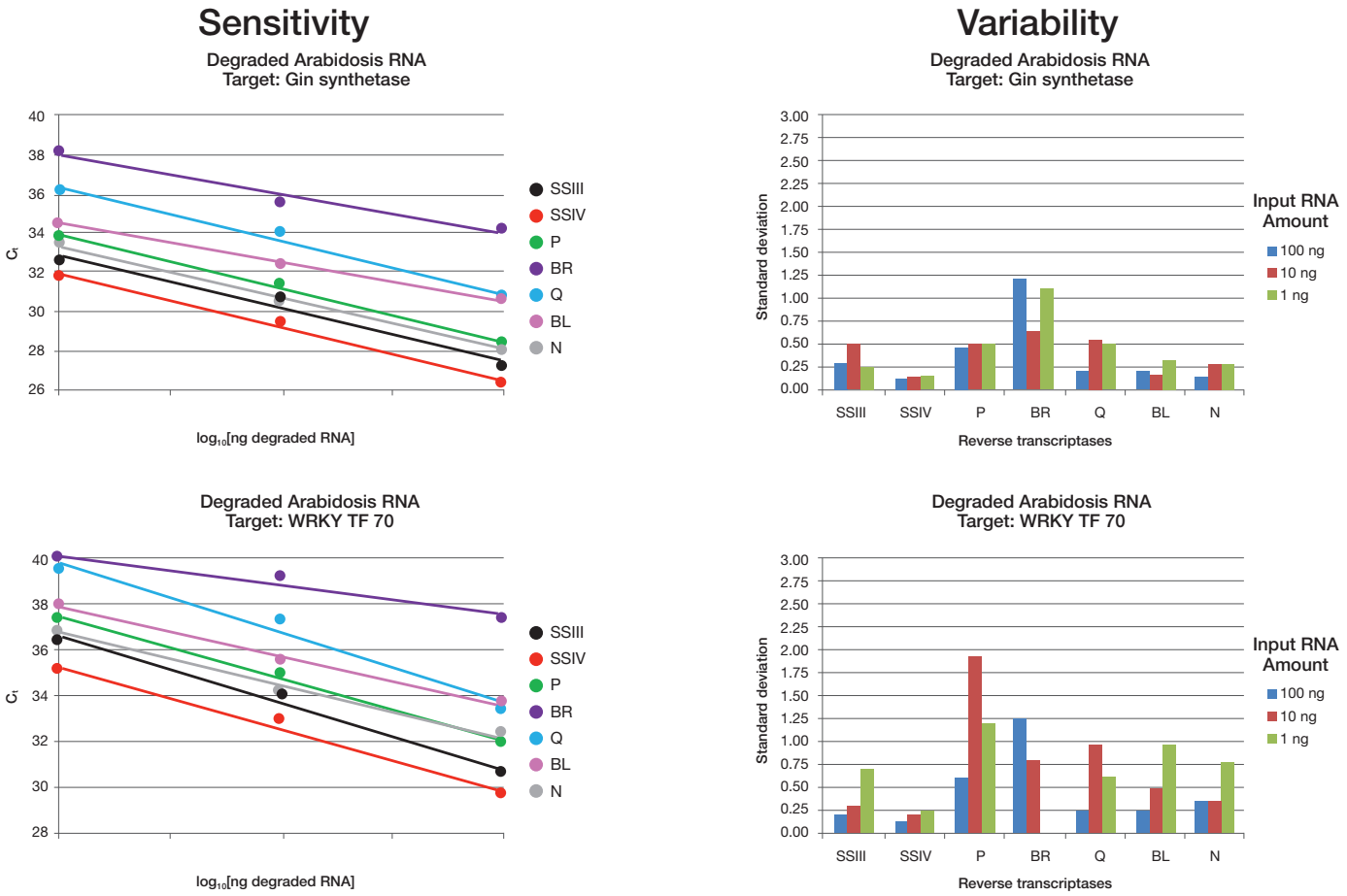

Figure 5. SuperScript IV RT and competitor RT sensitivity and variability with degraded plant RNA.

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 deviation 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.

SuperScript IV RT and competitor RT processivity study

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 speed and length of reverse transcriptases was assessed using a 0.5–9 kb RNA ladder. Reverse transcriptase reaction time was 10 minutes. 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 competitor enzymes failed to synthesize cDNA up to 3 kb, indicating that SuperScript IV RT is the most processive reverse transcriptase.

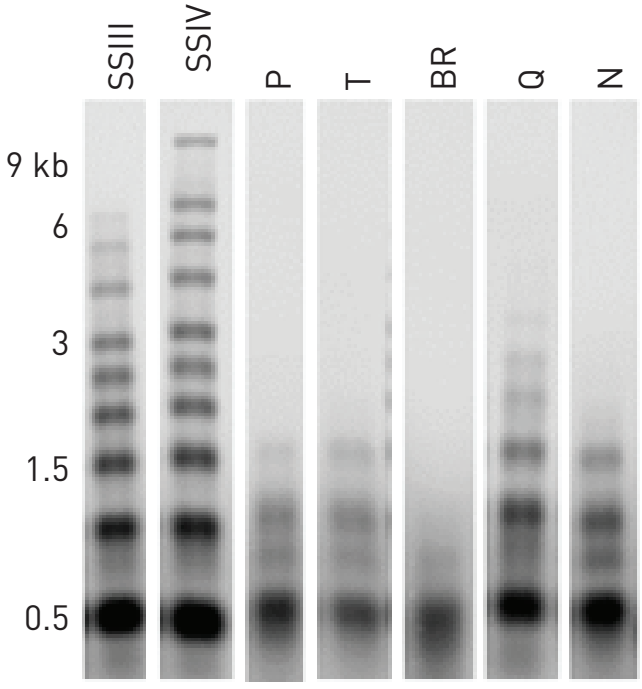

Figure 6. SuperScript IV RT and competitor RT processivity study.

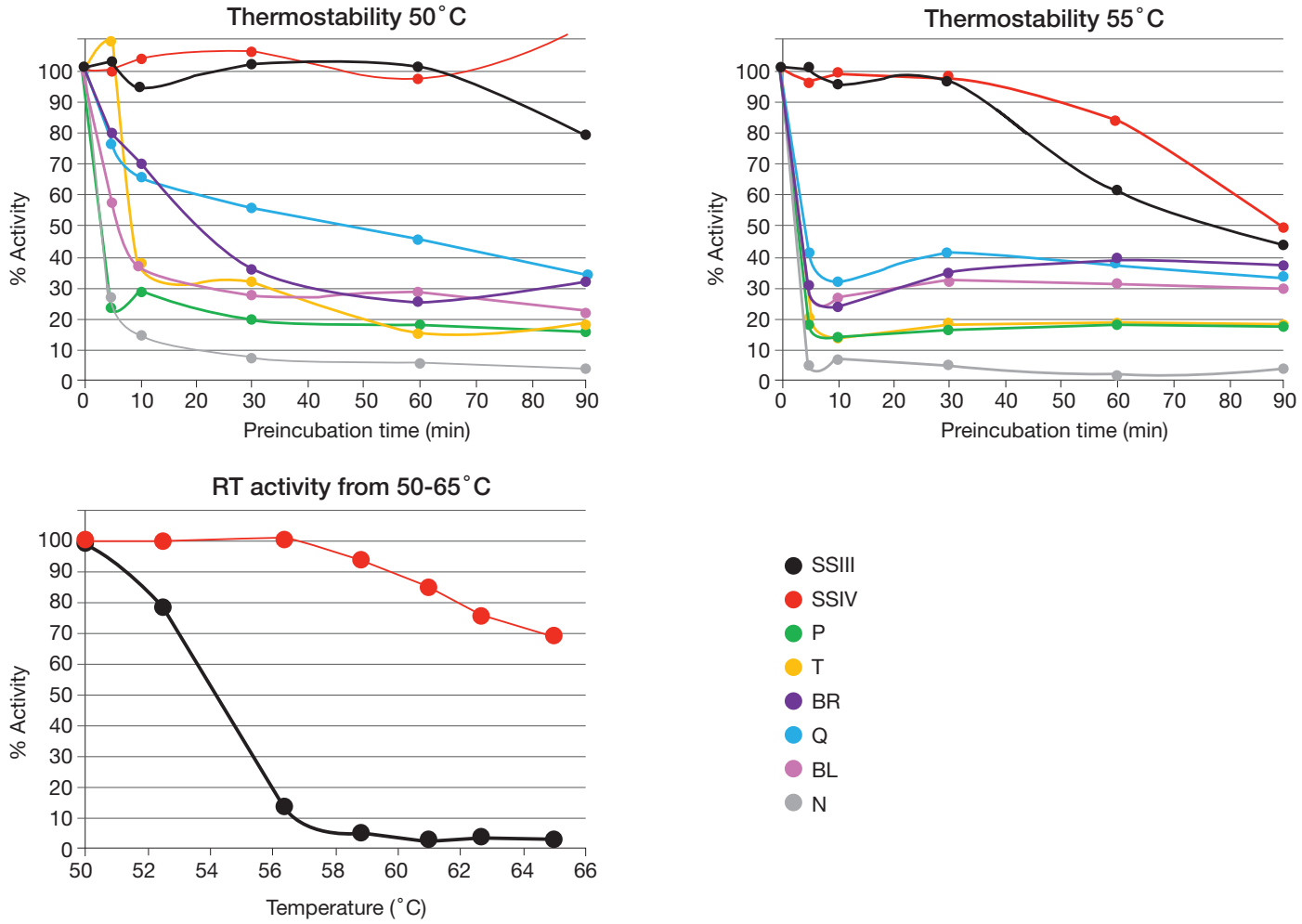

Figure 7. SuperScript IV RT and competitor RT thermostability study.

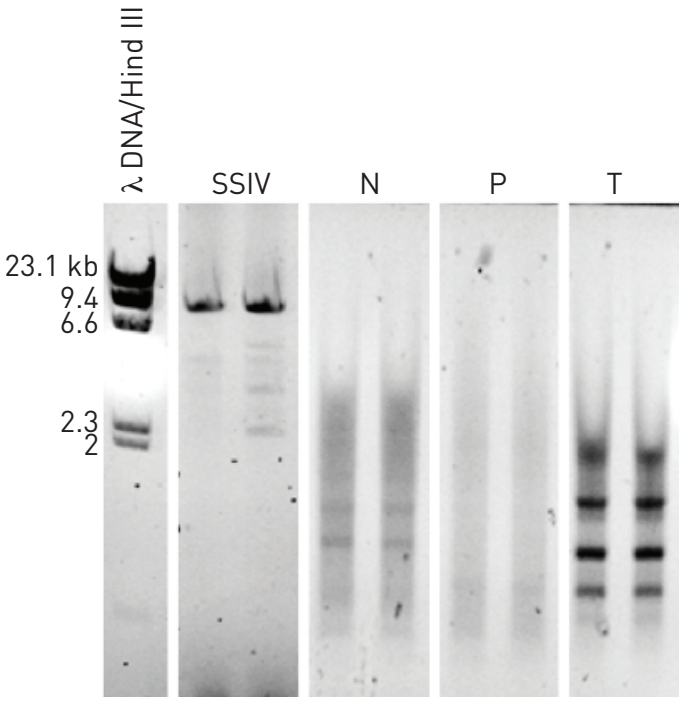

Figure 8. SuperScript IV RT and competitor RT cDNA length study.

SuperScript IV RT and competitor RT thermostability study

Thermostability of the reverse transcriptases was evaluated by preincubating at 50°C from 0 to 90 minutes. Following preincubation, polymerization activity was measured using a fluorescent activity assay. Only SuperScript III and IV RT remained active at 50°C for a sustained period of time (Figure 7). SuperScript III and SuperScript IV RT thermostability 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 yield and length of this RNA ladder dropped drastically with temperatures slightly above 50°C for SuperScript III RT. However, SuperScript IV RT sustains 100% activity up to 56.4°C and 70% activity up to 65°C. SuperScript IV RT's ability to function at higher temperatures enables the reverse transcription of RNA targets with strong secondary structure.

SuperScript IV RT and competitor RT cDNA length study

Although most processed RNAs are around 3 kb, there are genes that exceed this size. To evaluate the performance of reverse transcriptases 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 minutes at recommended reaction temperature, 15 minutes at 5°C above the recommended reaction temperature, and 15 minutes 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 in only the SuperScript IV RT reaction while all other reverse transcriptases produced smears and smaller products (Figure 8).

Conclusion

Scientific advancement requires researchers to continually undertake new challenges and obtain results more rapidly. Thermo Fisher Scientific strives to meet the needs of the scientific community by continuously evolving its 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.

Find out more at **lifetechnologies.com/ssiv**

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