

Improved RT-qPCR analysis of difficult plant RNA samples



Key findings

- Plant samples are considered difficult due to inhibitors and low RNA integrity
- Different reverse transcriptases have different abilities to work on plant samples
- Invitrogen™ SuperScript™ IV Reverse Transcriptase (RT) is the best reverse transcriptase tested due to its resistance to inhibitors, higher sensitivity with degraded RNA, and faster reaction times

Introduction

Extensive qualitative and quantitative feedback from plant researchers has indicated that most reverse transcriptase enzymes do not perform well in reactions with input from difficult samples, such as poorly purified RNA containing plant-specific inhibitors, degraded RNA, and unpurified RNA (direct lysates). Using intact RNA is critical for successful results in applications like reverse transcription quantitative PCR (RT-qPCR) to obtain reliable quantification of gene expression [1, 2]. Certain properties of plants such as a durable cell wall and metabolites including polysaccharides, phenolics, and flavonoids present obstacles against effectively purifying RNA [3]. The significant variability of cellular components that interfere with proper isolation of RNA is further complicated by differences in plant species, tissue types, environmental conditions, and developmental stages [3, 4]. An example of the varying integrity of RNA from different plant species can be seen when RNA from multiple plant types are extracted, purified, and compared using an RNA integrity number (RIN) algorithm (Figure 1).

To address the poor performance of reverse transcription from difficult plant samples, we have introduced Invitrogen™ SuperScript™ IV Reverse Transcriptase (RT). Experimental evidence shown here supports that SuperScript IV RT is the most robust reverse transcriptase compared to other commercially available enzymes for difficult plant RNA samples. Characterization of SuperScript IV RT was performed in non-ideal reaction conditions to address difficult RNA samples. Using a variety of stringent assays, this application note demonstrates that SuperScript IV RT possesses superior performance in the presence of a variety of plant-based inhibitors, such as polysaccharides, phenolics, and flavonoids along with typical sample preparation inhibitors including detergents, alcohols, salts,

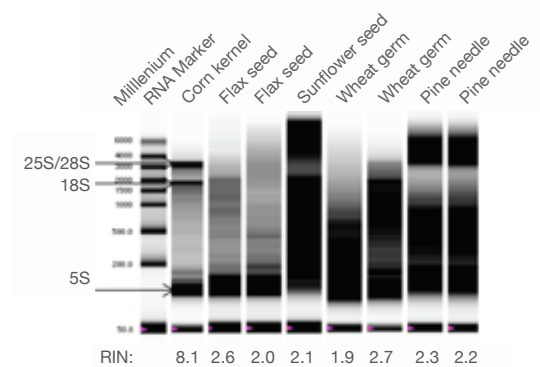


Figure 1. Comparison of purified RNA quality from various plant samples.

heparin, and hematin. The results show SuperScript IV RT maintained the highest sensitivity with both purified RNA (RIN: 1–3) and unpurified RNA samples. Furthermore, SuperScript IV RT exhibits the lowest variability with different amounts of input RNA. Finally, in 10 minutes SuperScript IV RT demonstrates superior inhibitor resistance and sensitivity when benchmarked against other leading reverse transcriptases following respective manufacturer protocols.

Materials and methods

RNA purification: *Arabidopsis* leaves, wheat germ tissue, and flax seed tissue purchased from the grocery store were ground to a fine powder in liquid nitrogen. RNA was extracted and purified using Invitrogen™ PureLink™ Plant RNA Reagent and the accompanying protocol. Total RNA was quantitated using the Thermo Scientific™ NanoDrop™ instrument and treated with Invitrogen™ DNase I. RNA quality was assessed using the Experion™ Automated Electrophoresis System (Bio-Rad) for RNA analysis and agarose gel electrophoresis with ethidium bromide staining.

Direct RNA extract preparation: *Arabidopsis* leaves, 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 buffer was added to the tube. The tube was then vortexed and centrifuged to pellet debris. The resulting clarified supernatant was transferred into a fresh microfuge tube and treated with DNase I.

Reverse transcription: 100 ng of purified RNA or 10% volume of direct RNA extract was reverse transcribed according to the SuperScript IV RT protocol or competitor protocols. SuperScript IV RT experiments were carried out in a 10-minute reaction.

qPCR: Reverse transcription reaction (cDNA) composed up to 10% of total qPCR reaction volume. Applied Biosystems™ TaqMan™ Assays for the gene targets are indicated in the figures. Invitrogen™ EXPRESS qPCR SuperMix and the Applied Biosystems™ ViiA™ 7 Real-Time PCR System were utilized. Direct RNA extract C_t values were reported with standard deviations to signify variability.

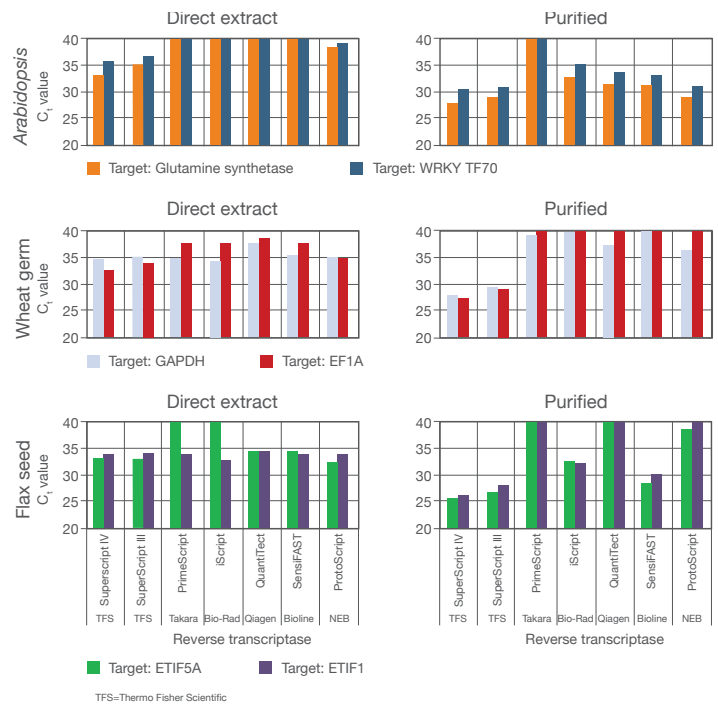


Figure 2. Comparing SuperScript IV RT and competitor RT sensitivity by qPCR analysis using direct RNA extracts or purified RNA. SuperScript IV RT, SuperScript III RT, and five competitor RTs were compared using either direct extracts of *Arabidopsis*, wheat germ, and flax seed RNA or 100 ng of the same purified RNA. Gene-specific TaqMan Assays were used for each plant species (*Arabidopsis*: glutamine synthetase and WRKY TF70; wheat germ: GAPDH and EF1A; flax seed: ETIF5A and ETIF1). Purified RNA RIN was ~2 for each plant sample (n = 3).

Results

Inhibitor resistance: SuperScript IV RT is the most sensitive reverse transcriptase when used with a variety of plant sample inhibitors, regardless of RNA purification process

Many plant researchers seek high-throughput methods to quickly identify viruses that harm plant populations [5]. To understand the accessibility of RNA for quantitation from non-purified samples, reverse transcription was performed using direct RNA extracts from common plant samples like *Arabidopsis*, wheat germ, and flax seed and compared to purified RNA samples from the same species.

When qPCR analysis was performed from cDNA generated using SuperScript IV RT, SuperScript III RT, and five competitor RTs using either direct RNA extract or purified RNA (Figure 2), the direct extracts showed considerable inhibition of the activity of all RT enzymes. These results are not surprising, considering the excess of a variety of inhibitors. Even so, SuperScript IV RT consistently exhibited lower C_t values for all targets compared to the other RTs when direct RNA extracts were used. With purified RNA, the results improve significantly for all 6 targets when

SuperScript IV RT is used (Figure 2). The purified RNA still challenged many of the competitor RTs, as even the purified RNA samples all had RIN values around 2. Use of SuperScript IV RT for both direct and purified RNA samples resulted in lower C_t values, or higher sensitivity, than other competitors in a variety of plant types.

Sensitivity and reproducibility: SuperScript IV RT has robust sensitivity and reproducibility compared to other leading RTs

Arabidopsis RNA was used in RT-qPCR studies to compare sensitivity and variability among SuperScript IV RT, SuperScript III RT, and five competitor RTs. TaqMan Assays for gene targets glutamine synthetase and WRKY TF70 were used to analyze cDNA from RT reactions using respective company RT protocols with random hexamers and a range of purified *Arabidopsis* RNA (1 ng, 10 ng, and 100 ng). For both targets, SuperScript IV RT was the most sensitive reverse transcriptase across a range of RNA concentration inputs, with lower C_t values compared to other leading RT competitors (Figure 3A and 3B). When standard deviations were calculated for each qPCR reaction triplicate, SuperScript IV RT had the lowest corresponding C_t variability, with a standard deviation equal to or below 0.2 (Figure 3C and 3D). The C_t value for the iScript™ cDNA

Synthesis Kit for target WRKY TF70 (1 ng RNA input) was undetermined ($C_t \geq 40$), therefore no standard deviation was reported.

Speed: SuperScript IV RT enzyme is faster than previous SuperScript RT enzymes

To fulfill the interest of plant researchers for a more robust SuperScript RT enzyme, the speed of reverse transcription was compared with the SuperScript RT family of enzymes: SuperScript II RT, SuperScript III RT, and SuperScript IV RT. The experiment was performed using a 10-minute RT reaction time, which is the standard in the SuperScript IV RT protocol. TaqMan Assays targeting GAPDH and EF1A for wheat germ, and ETIF5A and ETIF1 for flax seed were used for qPCR analysis of cDNA generated from each SuperScript enzyme reaction.

The results show SuperScript II RT and SuperScript III RT have comparable C_t values while SuperScript IV RT has significantly lower average C_t values for all plant samples and gene targets (Figure 4). With only a short 10-minute RT reaction, SuperScript IV RT is the most sensitive SuperScript enzyme compared to SuperScript II and III RTs. For plant-specific RNA samples, SuperScript IV RT outperforms previous SuperScript enzymes with its sensitivity and speed.

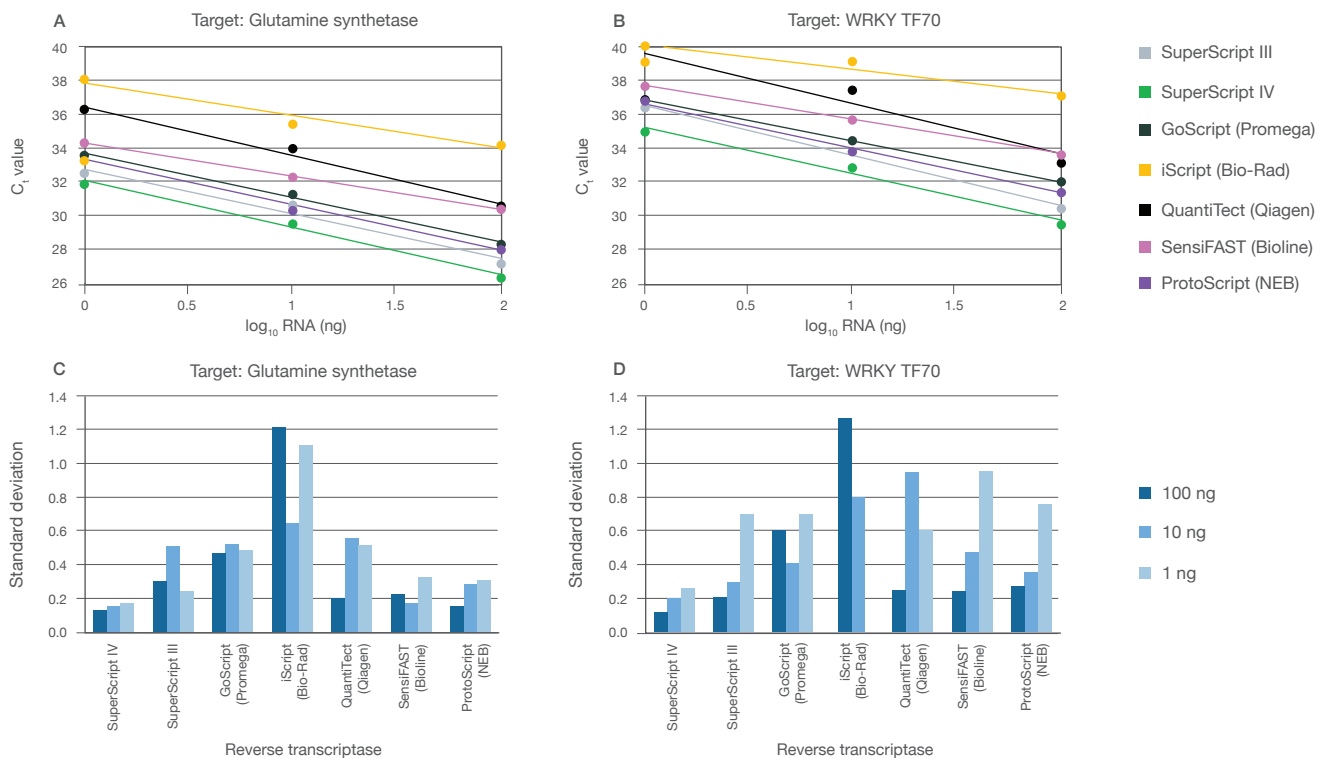


Figure 3. Comparing SuperScript IV RT and competitor RT sensitivity and variability with purified *Arabidopsis* RNA. Gene-specific TaqMan Assays targeting (A) glutamine synthetase and (B) WRKY TF70 were used for qPCR analysis of reverse transcription reactions with SuperScript IV RT, SuperScript III RT, and five competitor RTs. A range of purified *Arabidopsis* RNA inputs (1 ng, 10 ng, and 100 ng) were used for analysis. Standard deviations were determined from C_t values in A and B for each RNA input and enzyme, and graphed for (C) glutamine synthetase and (D) WRKY TF70. RIN for purified *Arabidopsis* RNA was ~2 (n = 3).

Conclusions

SuperScript IV RT enables plant scientists to quickly progress their research by providing sensitive and reliable reverse transcription from difficult samples that contain low copies of target RNA, poorly purified RNA, and inhibitors. Additionally, SuperScript IV RT provides the capability to directly analyze gene expression without RNA purification.

Many reverse transcriptases meet plant researcher expectations when used with high-quality RNA samples. Under conditions where inhibitors are present, RNA is degraded, and sample preparation is difficult, SuperScript IV RT is superior. Furthermore, SuperScript IV RT is a robust enzyme that demonstrates resistance to natural plant-derived inhibitors, shows higher sensitivity, and offers superior reproducibility in a fast 10-minute reaction time.

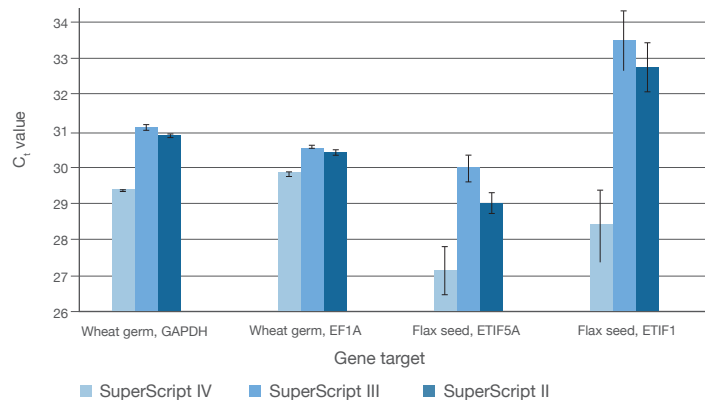


Figure 4. Comparison of SuperScript IV RT to SuperScript II RT and SuperScript III RT with purified wheat germ and flax seed RNA. Samples were purified using the PureLink Plant RNA Reagent protocol. Respective RT reaction protocols were followed using 100 ng RNA and random hexamers and an RT reaction time of 10 minutes. TaqMan Assays used in RT-qPCR targeted GAPDH and EF1A for wheat germ, and ETIF5A and ETIF1 for flax seed. RIN for purified RNA was ~2 (n = 3).

References

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Ordering information

Product	Quantity	Cat No.
SuperScript IV Reverse Transcriptase	2,000 units	18090010
SuperScript IV Reverse Transcriptase	10,000 units	18090050
SuperScript IV Reverse Transcriptase	4 X 10,000 units	18090200
SuperScript IV First-Strand Synthesis System	50 reactions	18091050
SuperScript IV First-Strand Synthesis System	200 reactions	18091200
PureLink Plant RNA Reagent	100 mL	12322-012
DNase I, Amplification Grade	100 units	18068-015
EXPRESS qPCR Supermix, universal	500 reactions	11785-200
EXPRESS qPCR Supermix, universal	2,500 reactions	11785-01K
ViiA 7 Real-Time PCR System with 384-well block	1 instrument	4453536

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