SuperScript IV VILO Master Mix for optimal RT-qPCR

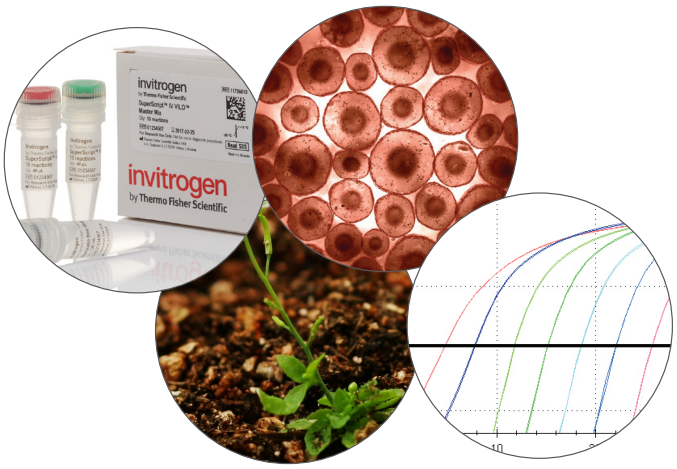
Abstract

The Invitrogen™ SuperScript™ product line provides market-leading reverse transcriptases (RTs) for the highest quality and reliability in cDNA synthesis. The newest enzyme in this family, Invitrogen™ SuperScript™ IV RT, is a thermostable enzyme that maintains the properties of high processivity and sensitivity even with challenging RNA samples. The new Invitrogen™ SuperScript™ IV VILO™ Master Mix is a first-strand cDNA synthesis master mix designed to transcribe from variable input of RNA to produce linear output of cDNA in two-step reverse transcription quantitative PCR (RT-qPCR). In this study, SuperScript IV VILO Master Mix was compared to the current Invitrogen™ SuperScript™ VILO™ Master Mix and other commercial cDNA synthesis products in an extensive variety of RT-qPCR applications. The results show that SuperScript IV VILO Master Mix delivers the highest yields, fastest reaction speed, increased sensitivity, and better performance with challenging RNA samples while maintaining high linearity and low variability across a broad range of input RNA amounts, giving researchers high confidence in their RT-qPCR results.

Introduction

Although the enzymatic properties of standard RTs, such as the RT from Moloney murine leukemia virus (M-MLV), have been adequate for basic reverse transcription applications, these enzymes do not offer high yields, sensitivity, speed, thermostability, or tolerance of challenging RNA samples. In RT-qPCR applications, the RT enzyme is not the only consideration for reliable results; the format of the kit used for cDNA synthesis is also important. For example, a one-tube master mix format reduces pipetting steps and handling of samples, reducing contamination concerns and improving reproducibility. An optimized master mix with a robust RT enzyme would improve RT-qPCR data consistency and subsequently reduce the number of repeat trials needed.

The original SuperScript VILO Master Mix was introduced to alleviate RT-qPCR analysis inconsistencies from variable RNA input; however, this master mix does not perform optimally with more challenging RNA samples and does not offer fast reaction times. SuperScript IV RT



(view the white paper) is a newly designed enzyme that allows very short reaction times, has improved sensitivity, and can perform well even with more challenging RNA samples (degraded or inhibitor-containing samples). Here we describe the benefits of combining SuperScript IV RT and the convenient 5X master mix VILO format for first-strand cDNA synthesis in two-step RT-qPCR applications. Where concern over genomic DNA (gDNA) contamination may be an issue, Invitrogen™ SuperScript™ IV VILO™ Master Mix with ezDNase™ enzyme allows optional gDNA removal. ezDNase enzyme is a novel DNase specific for double-stranded DNA that provides the fastest and gentlest method to remove gDNA from an RNA sample before cDNA synthesis. The results presented here demonstrate that SuperScript IV VILO Master Mix, used with both Applied Biosystems™ TaqMan® and SYBR™ Green qPCR assays, exhibits the best linearity, speed, sensitivity, and tolerance of challenging RNA samples, compared to SuperScript VILO Master Mix and other commercial cDNA synthesis master mixes. With its superior properties, SuperScript IV VILO Master Mix is the optimal choice for cDNA synthesis, helping to ensure the most accurate and reliable RT-qPCR results.

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Table 1. Reverse transcription reaction components and conditions by supplier.

Product	Number of separate master mix components to be mixed (except RNA and water)	Primers	Reaction setup	Reaction cycle times	Total cDNA synthesis reaction time
SuperScript IV VILO Master Mix	1 (5X master mix)	Random primers, oligo(dT) primers	5X master mix, RNA, water	25°C 10 min, 50°C 10 min, 85°C 5 min	25 min
SuperScript IV VILO Master Mix with ezDNase enzyme	3 (ezDNase enzyme, ezDNase buffer, 5X master mix)	Random primers, oligo(dT) primers	ezDNase enzyme, ezDNase buffer, RNA, water (2 min, 37°C); add 5X master mix	37°C 2 min, 25°C 10 min, 50°C 10 min, 85°C 5 min	27 min
SuperScript VILO Master Mix	1 (5X master mix)	Random primers	5X master mix, RNA, water	25°C 10 min, 42°C 50 min, 85°C 5 min	65 min
Supplier 1	1 (5X master mix)	Random primers, oligo(dT) primers	5X master mix, RNA, water	25°C 5 min, 42°C 20 min, 95°C 1 min	26 min
Supplier 2	1 (5X master mix)	Random primers, oligo(dT) primers	5X master mix, RNA, water	25°C 10 min, 42°C 60 min, 85°C 5 min	75 min
Supplier 3	4 (random primers, oligo(dT) primers, 2X reaction mix, 10X enzyme mix)	Random primers, oligo(dT) primers	Random primers, oligo(dT) primers, RNA, water (65°C 5 min); add reaction and enzyme mix	65°C 5 min, <1 min on ice, 42°C 60 min, 80°C 5 min	70 min
Supplier 4	7 (oligo(dT) primers, random primers, 5X reaction buffer, $MgCl_2$, PCR nucleotide mix, RNase inhibitor, RT enzyme)	Random primers, oligo(dT) primers	Random primers, oligo(dT) primers, RNA, water (70°C 5 min); add water, 5X reaction buffer, $MgCl_2$, PCR nucleotide mix, RNase inhibitor, RT enzyme	70°C 5 min, on ice 5 min, 25°C 5 min, 42°C 60 min, 70°C 15 min	90 min
Supplier 5	4 (first-strand 2X master mix, oligo(dT) primers, random primers, RT/RNase mix)	Random primers, oligo(dT) primers	First-strand 2X master mix, oligo(dT) primers, random primers, RT/RNase mix, RNA, water	25°C 5 min, 42°C 15 min, 95°C 5 min	25 min
Supplier 6	4 (7X gDNA buffer, RT enzyme mix, 5X RT buffer, RT primer mix)	Not specified	gDNA buffer, RNA, water (42°C 2 min); add RT enzyme mix, 5X RT buffer, RT primer mix	42°C 2 min, <1 min on ice, 42°C 15 min, 95°C 3 min	20 min
Supplier 7	2 (20X RT enzyme, 5X buffer)	Not specified	5X buffer, RT enzyme, RNA, water	25°C 5 min, 55°C 10 min, 85°C 5 min	20 min
Supplier 8	1 (5X master mix)	Random primers, oligo(dT) primers	5X master mix, RNA, water	25°C 5 min, 42°C 30 min, 85°C 5 min	40 min
Supplier 9	1 (5X master mix)	Random primers, oligo(dT) primers	5X master mix, RNA, water	37°C 15 min, 85°C 5 sec	15 min
Supplier 10	3 (5X master mix, DNase, DNase buffer)	Random primers, oligo(dT) primers	DNase buffer, DNase, RNA, water (25°C 5 min; 75°C 5 min); add 5X master mix	25°C 5 min, 75°C 5 min, 25°C 5 min, 46°C 20 min, 95°C 1 min	36 min

Materials and methods

- SuperScript IV VILO Master Mix (Cat. No. 11756050)
  - Components: SuperScript IV VILO Master Mix (5X), SuperScript IV VILO Master Mix no-RT control, nuclease-free water
- SuperScript IV VILO Master Mix with ezDNase enzyme (Cat. No. 11766050)
  - Components: SuperScript IV VILO Master Mix (5X), SuperScript IV VILO Master Mix no-RT control, ezDNase enzyme, ezDNase buffer (10X), nuclease-free water

RNA purification

RNA was extracted from frozen human lung tissue and purified using the Invitrogen™ PureLink™ RNA Mini Kit (Cat. No. 12183018A) according to the standard protocol. Total RNA was quantitated by Thermo Scientific™ NanoDrop™ spectrophotometer. RNA quality was assessed using the Bio-Rad™ Experion™ Automated Electrophoresis System and agarose gel electrophoresis with ethidium bromide staining.

RNA preparation with inhibitors

For analyzing the effect of inhibitors on reverse transcription, Invitrogen™ Cervical Adenocarcinoma (HeLa-S3) Total RNA (Cat. No. AM7852) was premixed with the following inhibitors: LiCl, Invitrogen™ TRIzol™ Reagent, SDS, heparin, and hemin. The final concentration of each inhibitor in the reverse transcription reaction is indicated in the results section.

Removal of gDNA with DNases

To determine the efficiency of gDNA removal, 100 ng of human gDNA (noncommercial) was spiked into 250 ng of HeLa total RNA and treated with either Invitrogen™ ezDNase enzyme (Cat. No. 11766051) or Invitrogen™ DNase I, Amplification Grade (Cat. No. 18068015). For ezDNase enzyme, RNA/DNA mixtures were incubated for 2 min at 37°C with 2 U of ezDNase enzyme and ezDNase buffer in 10 µL reactions. After incubation, the samples were added directly to reverse transcription reactions. For DNase I, RNA/DNA mixtures were incubated for 20 min at 37°C with 1 U of DNase I and reaction buffer in 10 µL reactions followed by a 10 min inactivation treatment with EDTA at 65°C. To evaluate the effect of DNase on RNA integrity in RT-qPCR, 100 ng of HeLa total RNA was pretreated with either ezDNase or DNase I enzyme and then diluted and used as input in duplicate reverse transcription reactions.

Reverse transcription

Reverse transcription was performed in 20 µL reactions using SuperScript IV VILO Master Mix (Cat. No. 11756050), SuperScript IV VILO Master Mix with ezDNase enzyme (Cat. No. 11766050), or cDNA synthesis products from other suppliers according to the instructions from each supplier (Table 1). First-strand synthesis reactions were performed on MJ Research thermal cyclers, Applied Biosystems™ ProFlex™ PCR System, or Applied Biosystems™ GeneAmp™ PCR System 9700.

TaqMan and SYBR Green qPCR assays

All parameters for optimizing the first-strand cDNA synthesis conditions were subsequently tested by qPCR with primer sets for various gene targets (indicated in figures) using TaqMan or SYBR Green assays. Samples were quantified with an Applied Biosystems™ ViiA™ 7 Real-Time PCR System (Cat. No. 4453536) using the default cycling protocols in Table 2. Invitrogen™ EXPRESS qPCR Supermix, Universal (Cat. No. 1178501K) was used for TaqMan assays and Invitrogen™ EXPRESS SYBR™ GreenER™ qPCR SuperMix, Universal (Cat. No. 1178401K) was used for SYBR Green assays. The SuperScript IV VILO Master Mix no-RT control was used as the negative control.

Reverse transcription reactions were prepared according to the user manual and run with suggested cDNA synthesis conditions. Afterwards, a volume of reverse transcription reaction equal to 10% of the qPCR reaction volume was added to subsequent TaqMan assays. For SYBR Green assays, depending on the starting amount of total RNA (e.g., 1 µg for 96-target panel), a 1:10 dilution of cDNA is required prior to adding 10% of the qPCR volume. For the gDNA removal experiment, RT samples were diluted 1:50 before qPCR. For the RNA integrity experiment, a 1:100,000 dilution of cDNA for eukaryotic 18S rRNA target was used. C_t values were normalized where indicated in the figures.

Table 2. qPCR cycling protocol using TaqMan and SYBR Green assays.

Temperature	Time	Step*	Cycles
50°C	2 min	UDG incubation	1
95°C	2 min	Polymerase activation	1
95°C	15 sec	Denaturation	40
60°C	1 min	Annealing/extension	

* A dissociation curve was run after all SYBR Green qPCR reactions.

Results and discussion

High linearity across a wide dynamic range of input RNA

The hallmark of VILO™ technology is the ability to accurately measure lower-copy targets as well as higher-copy targets from variable total RNA input by RT-qPCR. To test whether SuperScript IV VILO Master Mix can also transcribe RNA to cDNA in a linear fashion, its performance was compared to SuperScript VILO Master Mix using HeLa RNA input from 1 µg down to 10 pg. Reactions with SuperScript IV VILO Master Mix maintained high linearity across the entire range of RNA input for both a higher-abundance target and a lower-abundance target (Figure 1). For the more abundant target, GAPDH (Figure 1A), reactions with SuperScript IV VILO Master Mix had a slope of –3.3, efficiency of 102.1%, and R^2 of 0.994, with C_t values lower than reactions with SuperScript VILO Master Mix. In a TaqMan assay with a less abundant target, PolE (Figure 1B), SuperScript IV VILO Master Mix had a slope of –3.36, efficiency of 98.6%, and R^2 of 0.990, with C_t values at all RNA inputs comparable to or lower than those of SuperScript VILO Master Mix. Overall, these results suggest great linearity with both master mixes and better yields with SuperScript IV VILO Master Mix than with the current SuperScript VILO Master Mix.

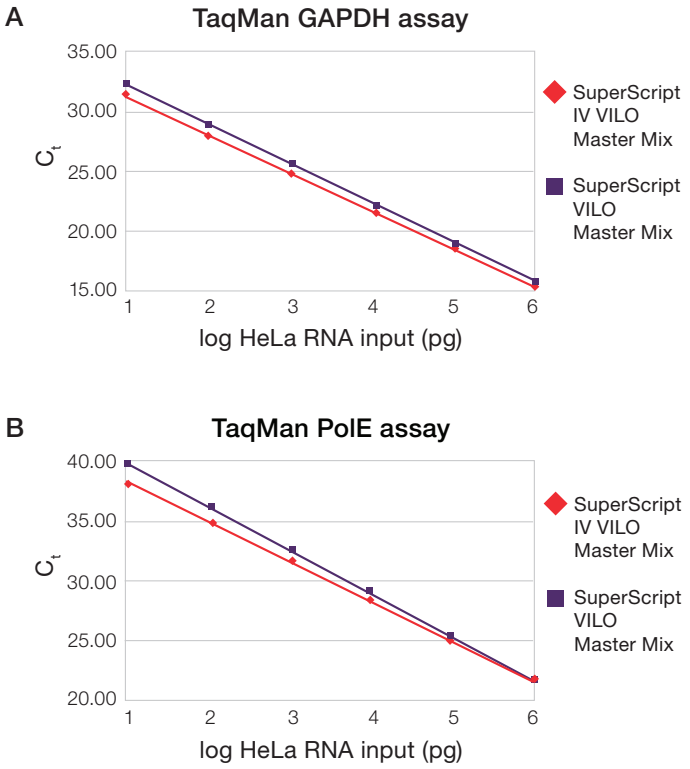

Figure 1. Linearity of SuperScript IV VILO Master Mix across different HeLa total RNA input for high-abundance (A) and low-abundance (B) gene target.

More cDNA faster than with other commercial kits, even at higher reaction temperatures

The high processivity of SuperScript IV RT in SuperScript IV VILO Master Mix allows completion of the RT reaction step in only 10 min. While some other commercial RT mixes also have relatively short protocols (less than 25 min), the short total reaction time does not guarantee better yield. When SuperScript IV VILO Master Mix was compared to SuperScript VILO Master Mix and 9 other commercial cDNA synthesis kits, SuperScript IV VILO Master Mix produced the highest cDNA yields for 14 targets in TaqMan assays using 1 ng of HeLa total RNA input (Figure 2, normalized fold change = 2^(C_t SuperScript IV VILO Master Mix – C_t other product)). This confirms that the highest cDNA yields are achievable in only 10 min with SuperScript IV VILO Master Mix.

Increasing the reverse transcription reaction temperature is sometimes recommended to help reduce RNA secondary structure that may interfere with the cDNA synthesis of certain targets (e.g., GC-rich targets). However, the increase in reaction temperature can reduce RT enzyme stability, leading to overall lower cDNA yield. To determine the effect of higher reaction temperature on the efficiency

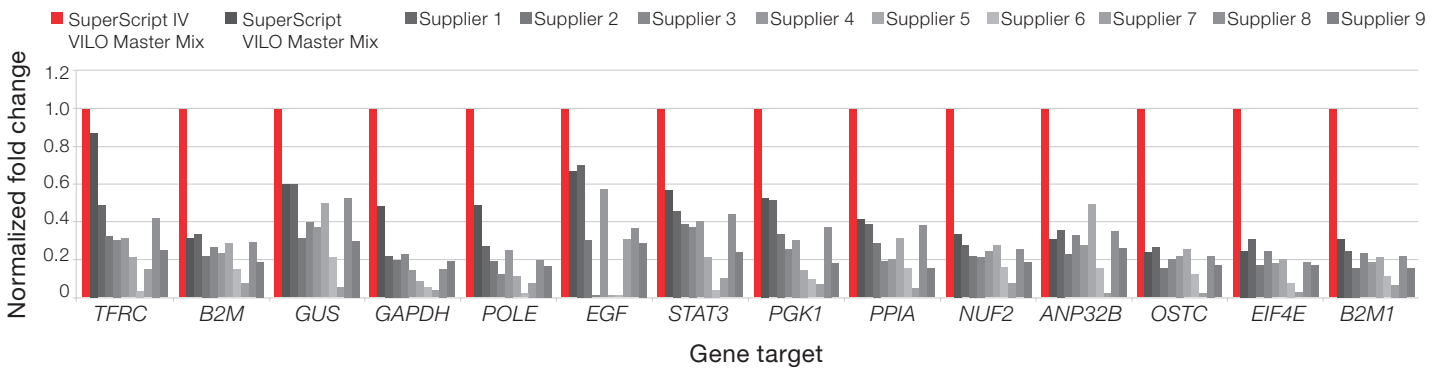

Figure 2. Higher cDNA yields with SuperScript IV VILO Master Mix than with other commercial kits.

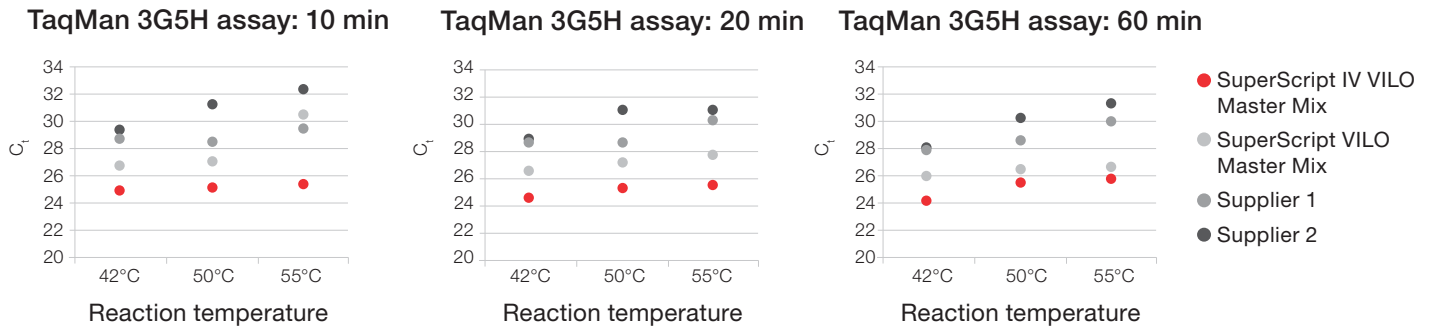

Figure 3. Higher thermostability of SuperScript IV VILO Master Mix compared to other commercial kits.

of RT enzymes, SuperScript IV VILO Master Mix, SuperScript VILO Master Mix, and two other suppliers' master mixes were compared using several reaction times (10 min, 20 min, and 60 min) and temperatures (42°C, 50°C, and 55°C) (Figure 3). When the reaction temperature was raised to 55°C, the C_t values increased significantly for the other master mixes, indicating these RT enzymes are less resilient than SuperScript IV VILO Master Mix to the higher temperatures. SuperScript IV VILO Master Mix maintains an average C_t value of 25.26 ± 0.48 for all times and temperatures tested, compared to average C_t values of 27.24 ± 1.28 for SuperScript VILO Master Mix, 29.04 ± 0.76 for the first supplier's product, and 30.53 ± 1.31 for the second supplier's product. These data show that SuperScript IV VILO Master Mix helps ensure the highest cDNA yields in the shortest time, even at elevated reaction temperatures.

Low variability and high sensitivity

Variability in RT-qPCR data caused by the reverse transcription step of the workflow can be reduced by using a master mix format, which obviates the need for extra handling and enables higher throughput. However, the master mix formulation must be robust enough to maintain consistent cDNA yield across technical and biological replicates for best data reproducibility. To investigate the variability in cDNA yields obtained with different cDNA synthesis kits for two-step RT-qPCR, 10 reverse transcription reactions using 100 pg of HeLa total RNA were performed with SuperScript IV VILO Master Mix, SuperScript VILO Master Mix, or another supplier's product. Triplicate qPCR reactions were performed for each reverse transcription reaction, using TaqMan GAPDH or PolE assays. When the 30 cumulative RT-qPCR data points were analyzed for the GAPDH target for each master mix, reactions with SuperScript IV VILO Master Mix had the lowest average C_t and similar standard deviation (28.10 ± 0.30) compared to reactions with SuperScript VILO Master Mix (29.16 ± 0.33) and the other supplier's product (30.41 ± 0.26) (Figure 4A). The same RT-qPCR analysis of the PolE target showed that reactions with SuperScript IV VILO Master Mix had the lowest average C_t value and standard deviation (34.05 ± 0.40) compared to reactions with SuperScript VILO Master Mix (36.20 ± 0.58) and the other supplier's product (37.35 ± 0.55) (Figure 4B). These data suggest that in addition to high sensitivity, speed, and thermostability, reactions with SuperScript IV VILO Master Mix are very robust and produce minimum variability, for high-confidence RT-qPCR results.

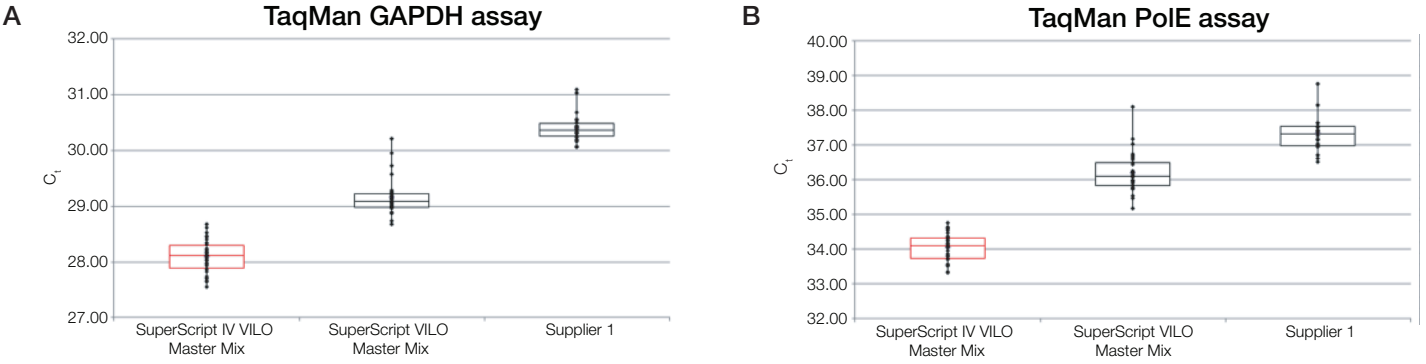

Figure 4. Assay variability and sensitivity with SuperScript IV VILO Master Mix and other commercial kits in (A) GAPDH and (B) PolE TaqMan assays.

Lower C_t values than with other commercial kits

Reported improvements to cDNA synthesis master mixes are often limited to a small subset of targets and RT-qPCR applications. To examine the capacity of SuperScript IV VILO Master Mix to transcribe RNA into cDNA for a broad range of targets, SuperScript IV VILO Master Mix was compared to SuperScript VILO Master Mix and other suppliers' master mixes using 96-gene TaqMan and SYBR Green assay panels with 100 ng HeLa total RNA (Figure 5). Using SuperScript VILO Master Mix as the reference, the ΔC_t values (ΔC_t = C_t SuperScript IV VILO Master Mix or other supplier's product – C_t SuperScript VILO Master Mix) are shown for each of 96 genes. With the SYBR Green assay panel, SuperScript IV VILO Master Mix had earlier C_t values than both alternative suppliers' products for 95 out of 96 targets, and for 80 out of 96 targets compared to SuperScript VILO Master Mix (Figure 5A). With the TaqMan assay panel, SuperScript IV VILO Master Mix had earlier C_t values than both alternative suppliers' products for 92 out of 96 targets and for 81 out of 96 targets compared to SuperScript VILO Master Mix (Figure 5B). In most cases, SuperScript IV VILO Master Mix lowered C_t values by more than 2 cycles compared to other commercial products, illustrating the improved efficiency and better yields of this master mix formulation (Figure 5A and B).

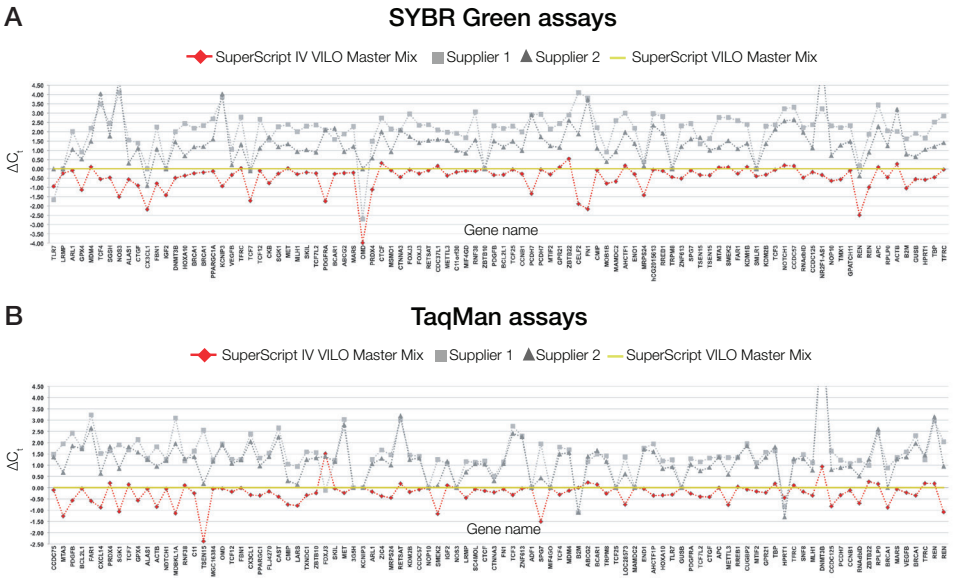

Figure 5. Performance of SuperScript IV VILO Master Mix and other commercial kits in 96-gene expression assays with (A) SYBR Green and (B) TaqMan assays.

More efficient than other master mixes with challenging samples

Degraded RNA poses a challenge for unbiased RNA-to-cDNA conversion, so the quality of RNA is important for reproducible and accurate RT-qPCR results. To determine if the improved robustness of SuperScript IV VILO Master Mix can alleviate challenges related to degraded RNA, 50 ng of RNA extracted from frozen lung tissue (RNA integrity number <5) was used to compare SuperScript IV VILO Master Mix to SuperScript VILO Master Mix and other suppliers' master mixes by TaqMan qPCR assays (Figure 6A). Analysis of 6 human targets showed that SuperScript IV VILO Master Mix is more efficient than all of the other tested products (Figure 6A, normalized fold change = 2^(C_t SuperScript IV VILO Master Mix – C_t other product)).

Another issue that contributes to inaccuracy in RT-qPCR is RT inhibitors that co-purify with the RNA sample during extraction and purification. Some examples include TRIzol Reagent, an RNA extraction reagent that contains phenol; SDS, a common solubilizing reagent; and salts such as lithium chloride (LiCl), used in multiple steps during RNA isolation and precipitation. In addition, RT inhibitors such as hemin and heparin may be inherent in the biological sample source. To test how these substances affect reverse transcription efficiency, several inhibitors were added to 100 ng of HeLa total RNA prior to the primer annealing step. The concentrations indicated in Figure 6B are the final concentrations of inhibitors in complete reverse transcription reactions. Also shown are control reactions without inhibitors (Figure 6B, no inhibitor). Reverse transcription with SuperScript IV VILO Master Mix, SuperScript VILO Master Mix, and seven other commercial master mixes followed by qPCR revealed that SuperScript IV VILO Master Mix is more efficient than SuperScript VILO Master Mix and other suppliers' products in the presence of all inhibitors tested (Figure 6B, normalized fold change = 2^(C_t SuperScript IV VILO Master Mix – C_t other product)). These data show that SuperScript IV VILO Master Mix is more efficient than other master mixes with challenging samples such as degraded or inhibitor-containing samples.

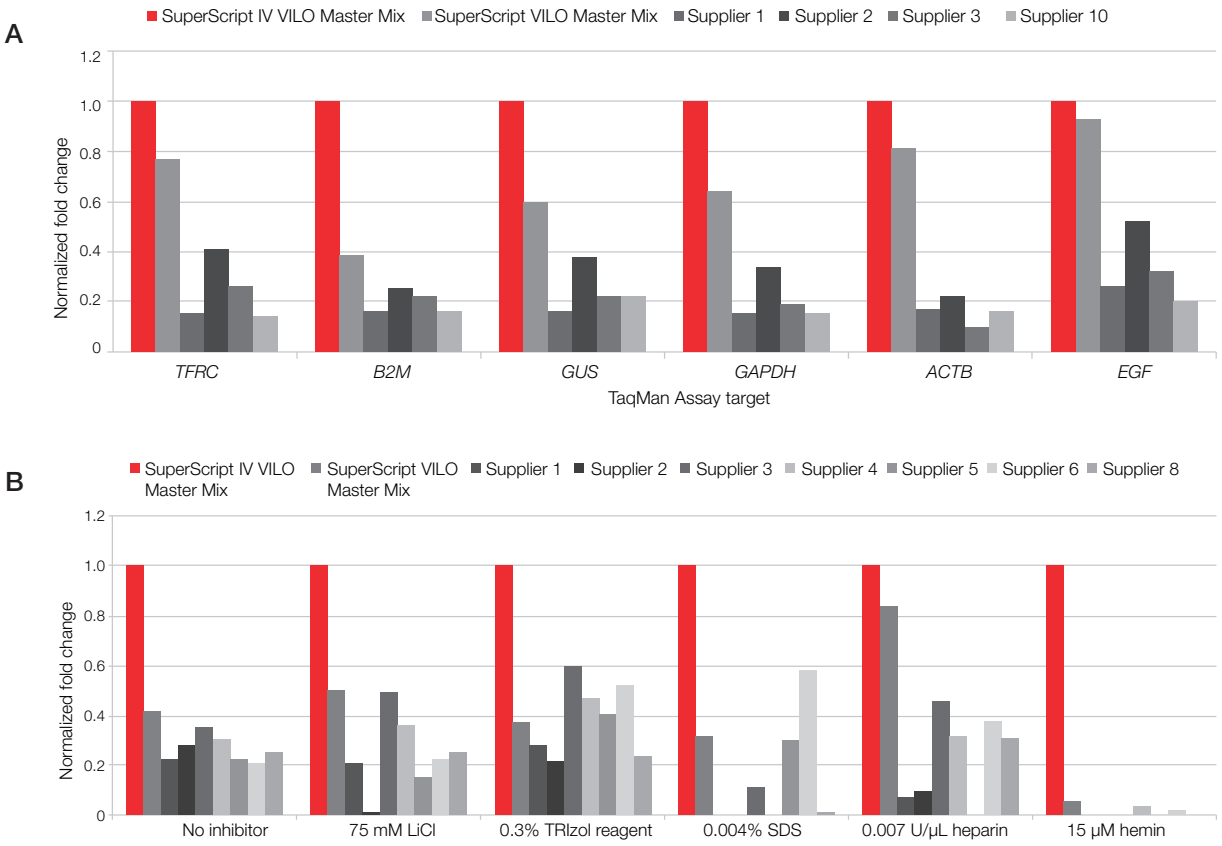

Figure 6. Better efficiency of SuperScript IV VILO Master Mix with (A) degraded and (B) inhibitor-containing RNA.

No-RT control and ezDNase enzyme for easy evaluation of gDNA contamination

Most current RNA purification methods fail to completely remove gDNA from RNA samples. Contaminating gDNA can lead to false-positive results as well as to more variability in RT-qPCR because it can affect specific amplification of target genes. To allow easy control over DNA contamination, SuperScript IV VILO Master Mix comes with a true no-RT control, which contains all of the master mix components minus the SuperScript IV enzyme. SuperScript IV VILO Master Mix with ezDNase enzyme also includes a double-stranded DNA–specific DNase enzyme for easy removal of DNA contaminants. The ezDNase enzyme protocol is a short treatment of RNA for 2 min at 37°C before primer annealing (Figure 7). To evaluate how the SuperScript IV VILO Master Mix no-RT control can indicate gDNA contamination, 100 ng of human gDNA was spiked into 250 ng of HeLa total RNA and mixed with the no-RT control. The sample was then treated with either ezDNase enzyme or DNase I and evaluated with a qPCR assay specific for human gDNA. Both ezDNase enzyme and DNase I effectively removed gDNA from the no-RT control, as shown by amplification plots of the RNA + gDNA + no-RT control sample before treatment and after treatment with either enzyme (Figure 8). However, compared to the traditional reverse transcription workflow with gDNA removal, SuperScript IV VILO Master Mix with ezDNase enzyme saves >70 min of reaction time (Figure 7).

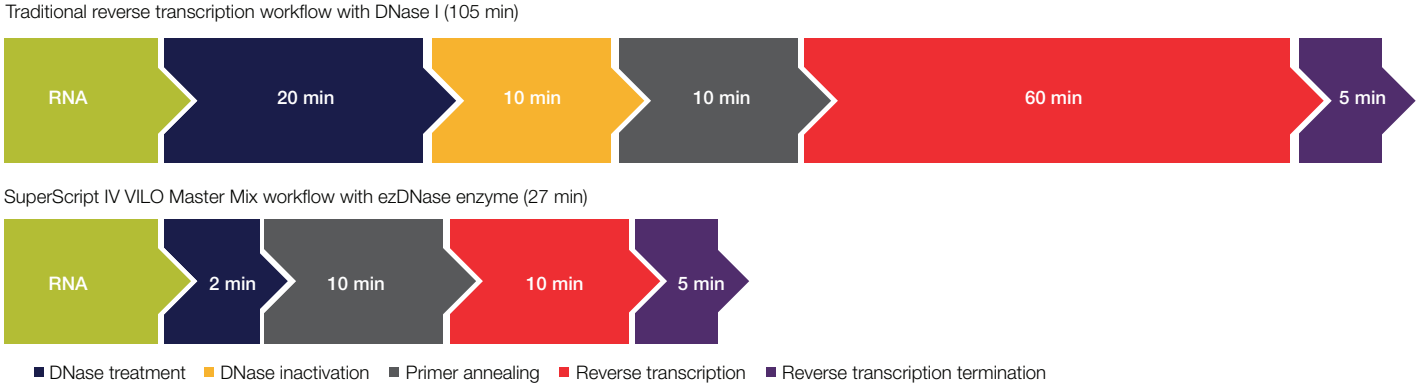

Figure 7. Comparison of traditional and SuperScript IV VILO Master Mix workflows with gDNA removal step.

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Safer gDNA removal and more accurate detection in RT-qPCR

While gDNA removal can be important for more accurate detection of specific targets using RT-qPCR, DNase treatment and inactivation can result in RNA sample damage or loss. In contrast to DNase I, which requires a special inactivation treatment that can have a negative effect on RNA integrity, ezDNase enzyme is easily inactivated at elevated temperatures and does not need to be removed prior to cDNA synthesis. Therefore, compared to DNase I, ezDNase enzyme is not only faster for gDNA removal but is also safer for the RNA. To test if RNA remains more intact with ezDNase enzyme, HeLa total RNA was treated with ezDNase enzyme and DNase I. Samples with ezDNase enzyme were immediately processed for RT-qPCR, while those with DNase I were first processed for DNase I inactivation according to standard protocols. Both RNA samples were serially diluted into duplicate RT-qPCR reactions with SuperScript IV VILO Master Mix and either TaqMan 18S rRNA assay or SYBR Green PBDG assay. Treatment with DNase I resulted in later C_t values (by 0.5 cycles on average) than treatment with ezDNase enzyme, for all RNA inputs except the lowest RNA input (Figure 9). These results suggest that DNase I treatment and inactivation does damage RNA integrity, and that with ezDNase enzyme treatment, more RNA is available for sensitive detection with SuperScript IV VILO Master Mix.

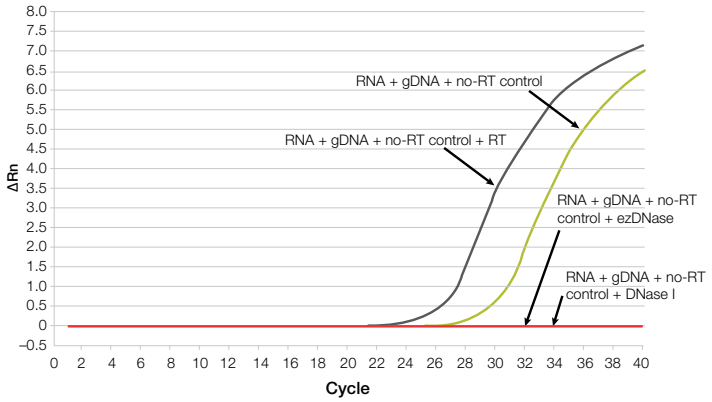

Figure 8. Amplification plots comparing ezDNase enzyme and DNase I for removal of gDNA added to the no-RT control.

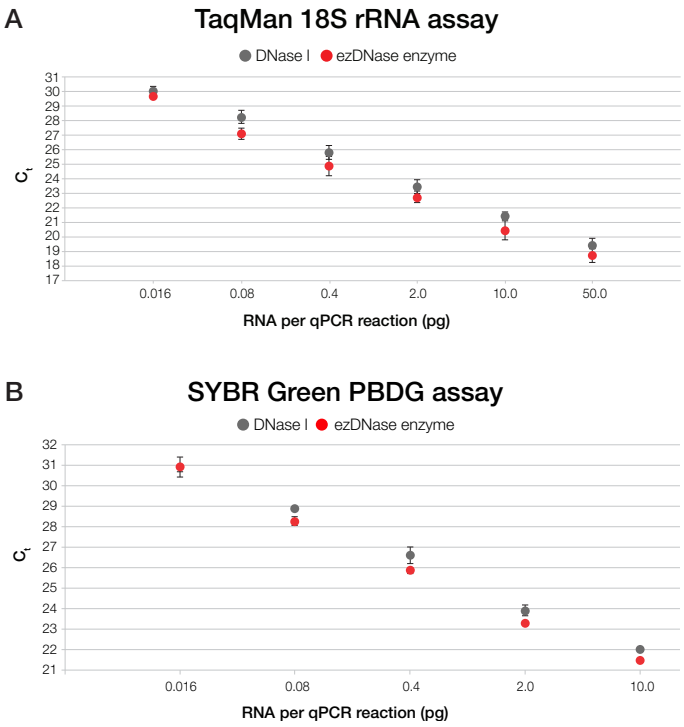

Figure 9. Integrity of RNA after treatment with ezDNase enzyme or DNase I, as measured by (A) TaqMan and (B) SYBR Green assays.

Conclusion

We constantly seek to enable scientists by not only providing the best reagents and tools for current and future research but also delivering these tools and reagents in the most user-friendly and reliable formats. As the data here shows, SuperScript IV VILO Master Mix is the superior cDNA synthesis master mix for two-step RT-qPCR. Combined with safe and easy integrated gDNA removal by ezDNase enzyme, SuperScript IV VILO Master Mix outperforms other cDNA synthesis kits and master mixes for two-step RT-qPCR in sensitivity, yield, and reproducibility for routine and challenging RNA samples with the shortest reaction time.

Find out more at **thermofisher.com/4vilo**

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