Reliable and sensitive RT-qPCR analysis of whole-blood RNA samples

Introduction

Most commercially available reverse transcription products do not perform well with difficult samples such as poorly purified, limited, or degraded RNA. As a consequence, studying gene expression by reverse transcription quantitative PCR (RT-qPCR) from challenging samples, including whole blood, is difficult and requires extensive optimization [1,2]. Since limited amounts of blood sample may be available, only a small quantity of total RNA may be used for RT-qPCR [3,4]. In addition, the collection, storage, and processing of blood samples can have negative effects on RNA integrity [5,6]. Another challenge is the presence of intrinsic substances that carry through with the extracted RNA and inhibit most common reverse transcriptases. These inhibitors include heme, anticoagulants (e.g., EDTA and heparin), and residual chemicals from the RNA extraction (e.g., guanidium thiocyanate) [7]. Complementary DNA (cDNA) synthesis from blood samples therefore demands a highly robust and sensitive reverse transcription reagent to produce cDNA of adequate quality and yield and to help ensure accurate and reliable RT-qPCR results.

We recently introduced Invitrogen™ SuperScript™ IV VILO™ Master Mix, a newly formulated first-strand cDNA synthesis master mix for two-step RT-qPCR. This master mix features the highly processive, extremely thermostable, and inhibitor-tolerant Invitrogen™ SuperScript™ IV Reverse Transcriptase in an optimized buffer and offers high efficiency, cDNA yields, and linearity in RT-qPCR applications (view the **white paper**). SuperScript IV VILO Master Mix offers a reaction time of only 10 minutes and allows cDNA synthesis at elevated temperatures for efficient reverse transcription of RNA with complex

secondary structures. The SuperScript IV VILO Master Mix format requires fewer pipetting steps, which helps scientists improve data reproducibility, avoid contamination, and reduce hands-on time.

This application note demonstrates that SuperScript IV VILO Master Mix offers efficient, reliable, and sensitive cDNA synthesis with low-quantity and low-quality RNA samples extracted from whole blood, for high-confidence RT-qPCR results.

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Materials and methods

Total RNA extraction from whole blood

Whole peripheral blood was collected in an EDTA tube, and total RNA was purified ~24 hours after collection using a protocol based on Invitrogen™ TRIzol™ Reagent (Cat. No. 15596026). Total RNA was quantified using a Thermo Scientific™ NanoDrop™ spectrophotometer, followed by genomic DNA removal.

RIN determination

The RNA integrity number (RIN) values of Invitrogen™ HeLa Total RNA (Cat. No. AM7852) and whole-blood total RNA were determined using the Experion™ RNA StdSens Analysis Kit (Bio-Rad, Cat. No. 7007103) on the Experion™ Electrophoresis Station (Bio-Rad, Cat. No. 7007010).

Reverse transcription

cDNA was synthesized from whole-blood total RNA at various concentrations, using SuperScript IV VILO Master Mix and four other commercial first-strand cDNA synthesis master mixes, following the manufacturers' protocols.

RT-qPCR

Synthesized cDNA constituting up to 10% of the total qPCR reaction volume was used with Invitrogen™ EXPRESS qPCR SuperMix (Cat. No. 1178501K). The RT-qPCR reactions were run on the Applied Biosystems™ ViiA™ 7 Real-Time PCR System (Cat. No. 4453536), and the threshold cycle (C_t) values were reported.

Results and discussion

More sensitive target detection from whole-blood RNA

Researchers have tested and used a range of preservation formats and purification protocols to extract and purify RNA from whole-blood samples. It has been shown that many of these approaches result in low RNA yield or integrity (Figure 1) [8,9]. When poor-quality RNA is used with non-robust reagents for gene expression analysis, resulting C_t values are often very high, results are unreliable, and gene expression is interpreted falsely [10,11]. We sought to evaluate how different reverse transcription reagents compare in their ability to provide reliable RT-qPCR data when challenged with low-quality RNA samples isolated from whole blood.

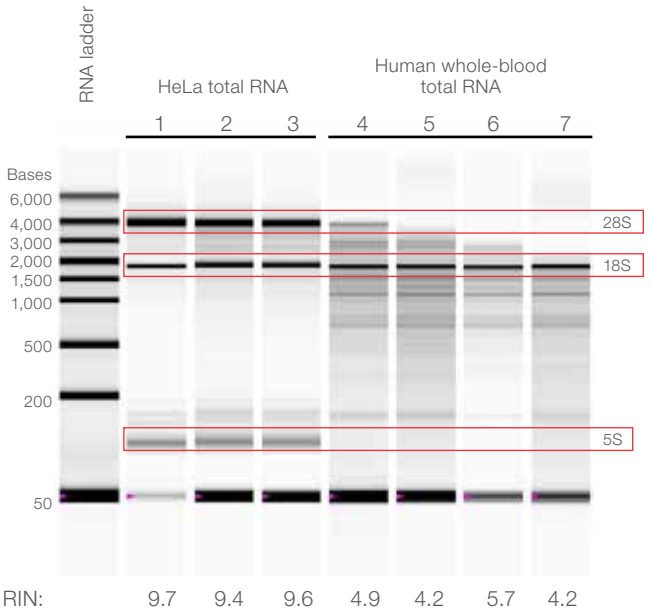

Figure 1. Comparison of HeLa RNA and human whole-blood RNA quality after a standard isolation procedure.

In the case of degraded RNA samples, high-quality and highly efficient reverse transcription reagents help ensure transcription of rare, intact RNA transcripts and allow qPCR analysis. To evaluate the ability of different reverse transcription master mixes to generate cDNA from low-quality RNA, RT-qPCR was performed using RNA isolated from whole blood (Figure 1, RIN 4.2–5.7) on ten gene targets. The targets included genes involved in cell signaling, such as *TGFB1* [12], as well as cytokine genes such as *IL18* and *TNF*, which play an important role in the innate immune response [13–15]. The results show that SuperScript IV VILO Master Mix was more efficient, based

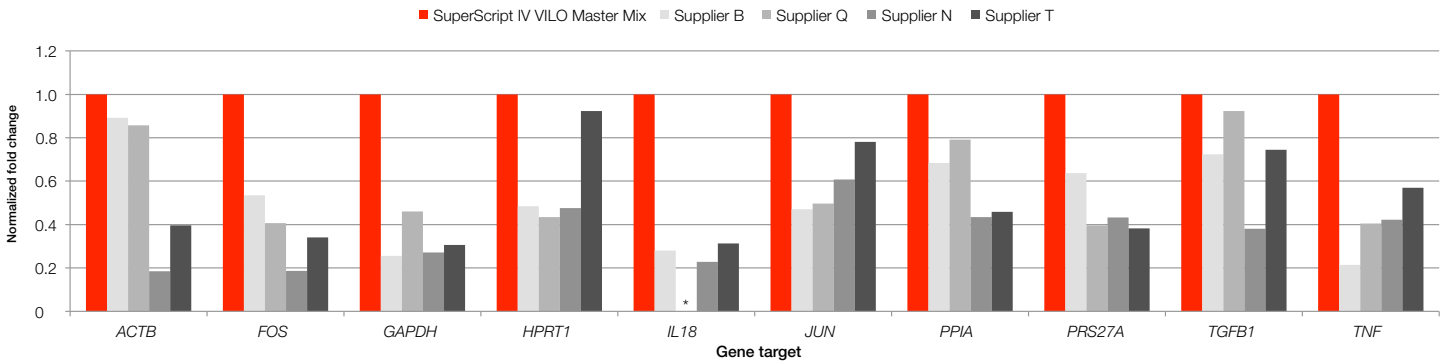

Figure 2. Highest efficiency with SuperScript IV VILO Master Mix on RNA from human blood. The efficiency of SuperScript IV VILO Master Mix as well as four other commercial first-strand cDNA synthesis products with degraded blood RNA samples was compared by RT-qPCR. Results are shown as normalized fold change relative to SuperScript IV VILO Master Mix, calculated as 2^(C_t SuperScript IV VILO Master Mix – C_t other product). *There was no C_t for *IL18* with Supplier Q's product.

on normalized C_t values, than all of the other commercial master mixes tested, with 1 ng of partially degraded RNA from whole blood (Figure 2). SuperScript IV VILO Master Mix was substantially more efficient at detecting *FOS*, *GAPDH*, *IL18*, and *TNF* targets than other master mixes, as shown by the lower normalized fold change of these targets with other master mixes (<0.6, Figure 2).

Further, to compare the sensitivity of different cDNA synthesis products, RT-qPCR assays were performed on a dilution series of low-quality whole-blood total RNA from 300 ng to 30 pg (Figure 3). The targets tested were the more-abundant *GAPDH* RNA and the less-abundant *PolE* RNA. SuperScript IV VILO Master Mix was more sensitive than the four other commercial products tested, and resulted in lower C_t values for both gene targets (Figure 3). SuperScript IV VILO Master Mix was the only master mix that was able to detect the less-abundant *PolE* target from as little as 300 pg of input RNA (Figure 3B). These results demonstrate that SuperScript IV VILO Master Mix offers highly sensitive and reliable detection of low-abundance gene transcripts even with a small amount of low-quality input RNA.

Reproducible results for high-confidence gene expression analysis

The RT step is often a major source of variation in RT-qPCR, due to the use of a non-robust reverse transcriptase, inhibition, and pipetting errors [16]. A master mix format with a robust enzyme can reduce reaction variability by decreasing the number of pipetting steps and alleviating inhibitor effects. We sought to explore the data reproducibility obtained with different cDNA synthesis master mixes with whole-blood RNA samples. Nine separate RT reactions were performed for each master mix with 100 ng of input total RNA from whole blood. qPCR

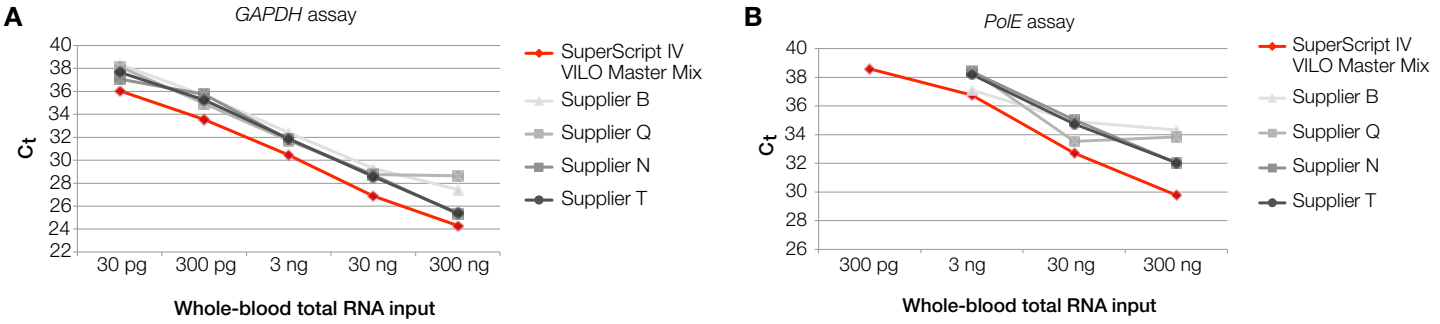

Figure 3. Highest sensitivity of SuperScript IV VILO Master Mix with limited and degraded RNA. RT-qPCR was performed on a dilution series of purified total RNA from whole blood, using SuperScript IV VILO Master Mix as well as four other commercial first-strand cDNA synthesis products, and Applied Biosystems™ TaqMan® Assays for **(A)** *GAPDH* and **(B)** *PolE*.

reactions were performed for each RT reaction using TaqMan Assays for *GAPDH*, *HPRT1*, and *PPIA*. For all three TaqMan Assays, SuperScript IV VILO Master Mix resulted in the lowest C_t values (*GAPDH:* 26.67 ± 0.1, *HPRT:* 31.1 ± 0.2, *PPIA:* 27.17 ± 0.14) (Figure 4). Furthermore, the range and standard deviation for all nine RT reactions tested on three TaqMan Assays were comparably low between SuperScript IV VILO Master Mix and the other master mixes (Figure 4). These results show that SuperScript IV VILO Master Mix offers better sensitivity while maintaining low variability for high-confidence RT-qPCR results.

Conclusions

SuperScript IV VILO Master Mix enables scientists working with suboptimal RNA samples, such as those from whole blood, to reliably evaluate gene expression, by providing highly robust reverse transcription in challenging conditions.

Many cDNA synthesis reagents can fulfill cDNA synthesis with optimal, high-quality, and abundant RNA samples. Under conditions where RNA is limited, degraded, or possibly contaminated with reaction inhibitors, SuperScript IV VILO Master Mix offers performance that other reagents cannot match. SuperScript IV VILO Master Mix is a cDNA synthesis master mix optimized for superior efficiency, sensitivity, and reproducibility in RT-qPCR applications.

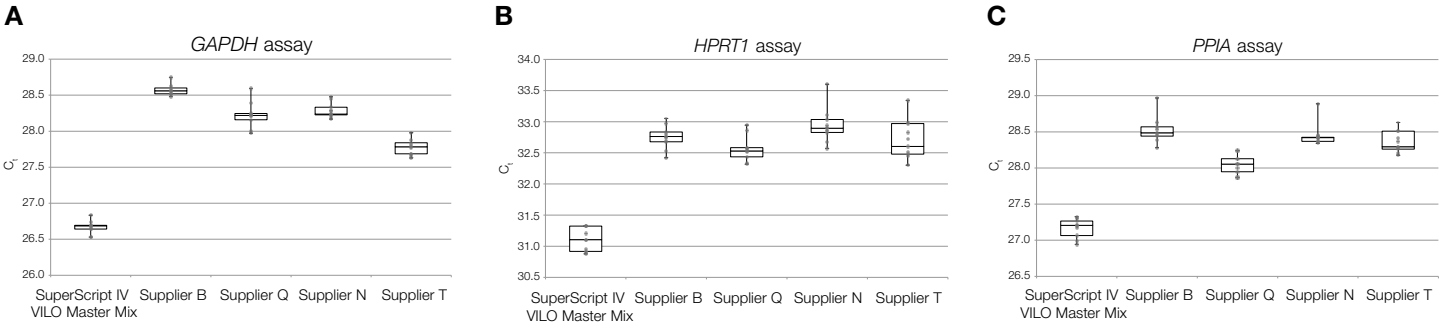

Figure 4. Low RT-qPCR variability with SuperScript IV VILO Master Mix. Nine separate RT-qPCR reactions were performed with SuperScript IV VILO Master Mix and four other commercial first-strand cDNA synthesis products, using 100 ng of purified total RNA from whole blood and TaqMan Assays for **(A)** *GAPDH*, **(B)** *HPRT1*, and **(C)** *PPIA* gene targets.

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Ordering information

Product	Quantity	Cat. No.
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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