Improved applications for RNA analysis

qScript Ultra, a novel, engineered, inhibitor tolerant reverse transcriptase enables rapid, full-length synthesis of cDNA at elevated temperatures

Ryan C. Heller and David Schuster

Quantabio, 100 Cummings Center Suite 407J, Beverly, MA 01915

Abstract

First-strand cDNA synthesis by a reverse transcriptase (RT) is an indispensable step that underpins many methods for RNA analysis including monitoring changes in gene expression levels, profiling noncoding RNA, RNA pathogen quantification and sequencing of full transcriptome or RNA viruses. Despite the pivotal role of RTs, most commercially available enzymes have limited speed, processivity, are adversely affected by sample matrix, or lack activity at temperatures sufficient to relax RNA secondary structure. These deficiencies typically result in incomplete and biased cDNA synthesis. Through a program of directed mutagenesis and enzyme variant screening, we have engineered a novel, inhibitor tolerant RNase H(-) RT called qScript Ultra that displays superior velocity and processivity with elevated activity over a broad range of temperatures.

Introduction

First-strand cDNA synthesis. For two-step RT-PCR approaches we developed the qScript Ultra Flex kit, which provides qScript Ultra RT in an optimized and stabilized master mix format and delivers efficient full-length cDNA synthesis with maximal yield at temperatures as high as 60°C. The enhanced velocity and processivity enables rapid and efficient conversion of long transcripts at elevated temperatures, critical for capturing RNA with high GC-content or regions of secondary structure. For RT-PCR applications that demand the highest consistency, reproducibility, and unbiased representation of the transcriptome in cDNA product we developed qScript Ultra cDNA SuperMix. Incorporating both anchored oligo(dT) and randomers, it contains all required components for cDNA synthesis except RNA template. The improved speed, processivity, and expanded thermal activity profile of qScript Ultra provides efficient linear conversion of RNA to cDNA in a 10-minute reaction at 55-60°C with exceptionally broad linear dynamic range.

One-step reverse transcription qPCR. For one-step, single-tube RT-PCR applications we developed optimized master mix formulations containing qScript Ultra RT, DNA polymerase, and a thermolabile uracil-DNA glycosylase for PCR carryover contamination control. In addition, a nucleic acid aptamer is included, effectively blocking RT activity at temperatures up to 35°C, yet allowing full RT activation at temperatures of 55-60°C. This highly stringent warm start feature is particularly important for allowing room-temperature reaction setup and for maintaining full specificity in complex multiplexed reactions. The robust, inhibitor tolerant characteristics of qScript Ultra allow for linear and consistent cDNA synthesis over a broad range of RNA input quantity and quality, including picogram levels of RNA and samples containing inhibitors derived from extraction chemicals, blood, plant, and animal tissues. Finally, qScript Ultra RT is also compatible with lyophilization, displaying full functional activity after drying and rehydrating, enabling formulations with extended stability at room temperature and allowing shipping without a requirement for dry ice and foam packaging.

Methods

Total human RNA samples used in this study was Agilent Universal Human Reference (UHR) RNA. First-strand cDNA synthesis reactions were performed using the qScript Ultra Flex kit or the qScript Ultra SuperMix. First-strand cDNA synthesis reactions were analyzed by qPCR using the PerfeCTa SYBR Green FastMix. 1-step RT-qPCR master mixes were compared using the CDC Influenza SARS-CoV-2 Multiplex assay[1]. Lyophilization of 1-step RT-qPCR master mix was done in 3 ml glass vials using a Millrock Stellar ST85 freeze dryer. Dahlia Latent Virioid ssRNA was prepared by in vitro transcription of dsDNA gene blocks (IDT), followed by DNase treatment and purification.

Conclusions

- The improved properties of the engineered qScript Ultra RT allow for increased reaction temperatures with high yields of long, > 17 kb cDNA products in only 10-minute reaction times.
- Optimized master mix formulations display broad RNA input compatibility with either flexibility in choice of priming method or the consistency and reproducibility of a single-tube SuperMix format.
- 1-step RT-qPCR formulations with qScript Ultra and warm-start aptamer are compatible with lyophilization and display high sensitivity and specificity and improved inhibitor tolerance. High RT temperatures enable sensitivity with difficult templates.

[1]https://www.cdc.gov/coronavirus/2019-ncov/lab/multiplex.html

[2]Giguère T, Perreault JP. Classification of the Pospiviroidae based on their structural hallmarks. PLoS One. 2017 Aug 4;12(8):e0182536. doi: 10.1371/journal.pone.0182536. PMID: 28783761; PMCID: PMC5544226.

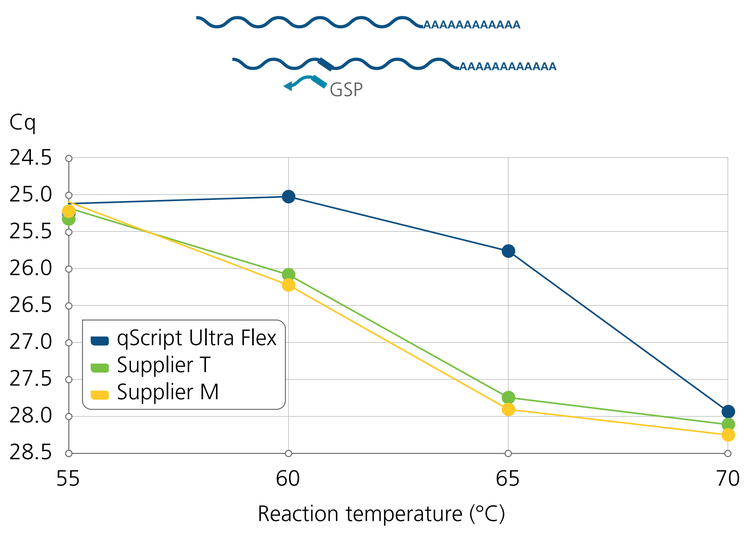

Figure 1 **Thermostability and Activity at High Temperatures.** cDNA was synthesized in reactions using 5 ng total human RNA and a stable LDHA gene-specific reverse primer. Reactions were incubated at the indicated temperatures, followed by termination and product detection by qPCR. The lower Cq values for the engineered qScript Ultra reverse transcriptase indicate more efficient cDNA synthesis at high temperatures up to 65°C, with no loss in performance up to 60°C, critical for progressing through high GC-regions and regions of RNA secondary structure.

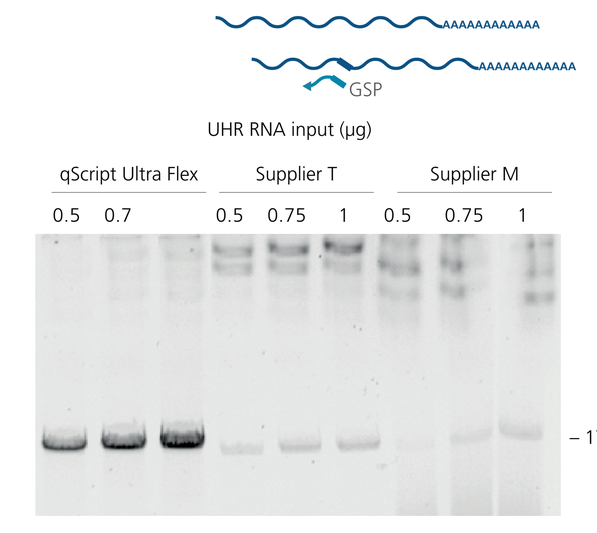

Figure 2 **Long Range cDNA Synthesis Capabilities.** cDNA synthesis reactions were carried out at the manufacturer recommended temperature of 55°C for 10 minutes for each enzyme system, and then a portion was used for endpoint PCR using the repliQa HiFi ToughMix with primers targeting a 17.7 kb portion of the human SYNE1 mRNA. The quantities of human total UHR RNA used for each reaction are indicated above each lane. A portion of the PCR products were then analyzed by agarose gel electrophoresis, showing high yields of full-length product with the qScript Ultra RT.

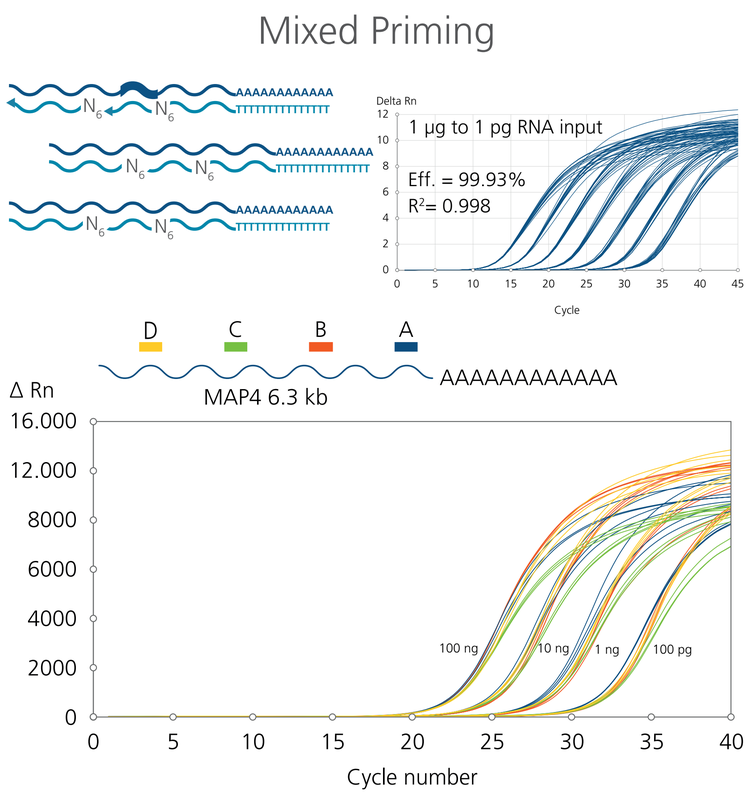

Figure 3 **Broad Input Range and Even Coverage with the qScript Ultra SuperMix.** Using a mixed priming strategy with the qScript Ultra SuperMix, indicated quantities of total human UHR RNA were reacted for 2 minutes at 25°C and 10 minutes at 55°C. A portion of the products were then analyzed by qPCR. **A**, Using an ACTB mRNA assay for analysis, results show a linear amplification response from 1 µg to 1 pg total RNA input in the cDNA synthesis reaction. **B**, Four qPCR assays were designed targeting different regions of the large MAP4 mRNA. Overlapping amplification curves indicate even coverage throughout the long transcript.

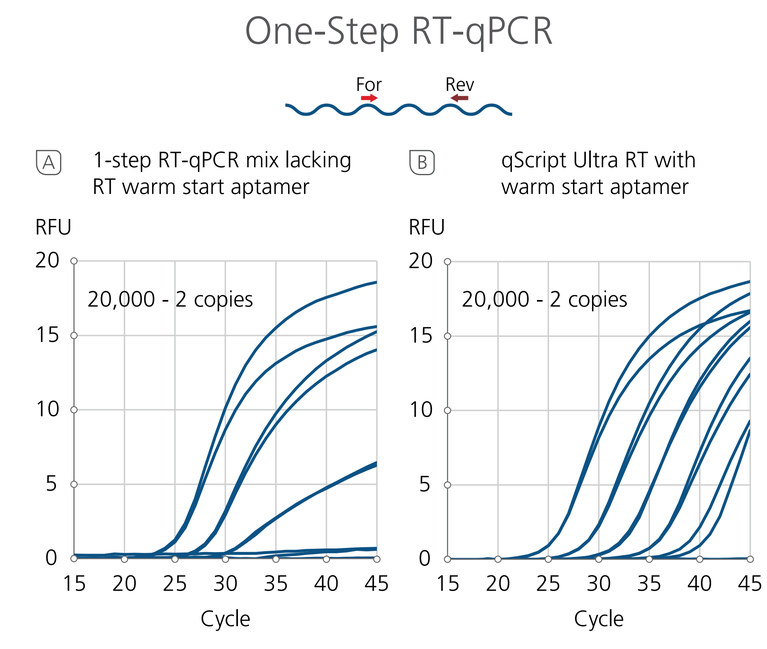

Figure 4 **Warm Start Aptamer in 1-step RT-qPCR.** Using 1-step RT-qPCR master mixes containing all components for cDNA synthesis and qPCR, indicated quantities of synthetic viral RNA controls were set up at room temperature, then reacted and analyzed using the CDC's Influenza SARS-CoV-2 Multiplex Assay[1]. The plots shown are from the FluA-FAM channel. **A**, Compared to a master mix lacking an RT warm-start feature, a qScript Ultra RT mix containing a warm-start aptamer (**B**) is showing significantly improved specificity.

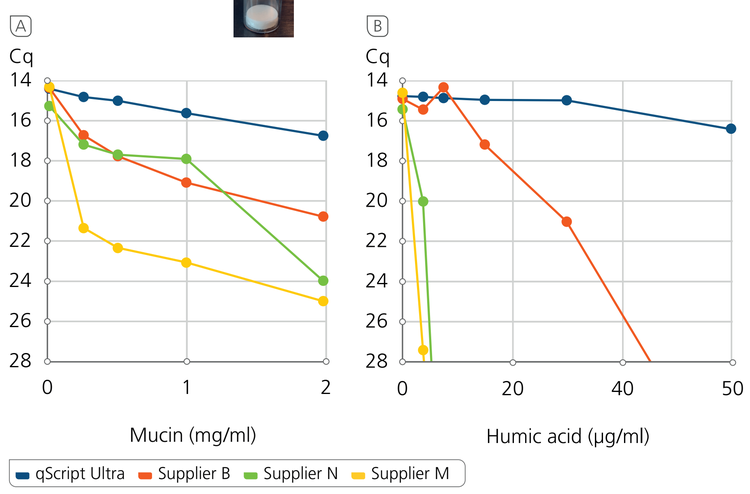

Figure 5 **Improved Inhibitor Tolerance in Lyophilized 1-step Formulation.** qScript Ultra 1-step mix was formulated with excipients and dried into an ambient-stable cake format. After rehydration, 50 ng of total human RNA was reacted with the qScript Ultra 1-step mix or with rehydrated or lyo-ready mixes from other suppliers and incubating according to manufacturer recommendations. Reactions contained a GAPDH primer assay and the indicated quantities of mucin (**A**) or humic acid (**B**).

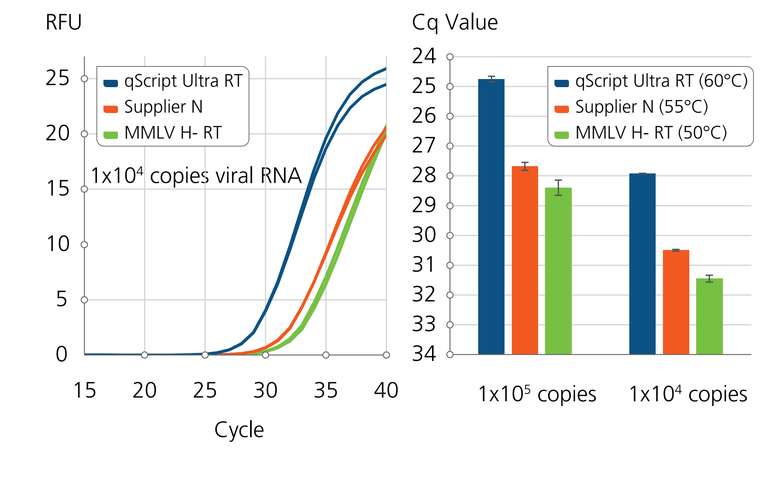

Figure 6 **1-step Amplification of a Virioid RNA with Stable Secondary Structure.** A Dahlia Latent Virioid RNA template was prepared by in vitro transcription of a dsDNA construct. The ssRNA molecule adopts a stable rod-like structure[2]. 1-step RT-qPCR reactions were assembled and incubated at the indicated RT temperature prior to thermocycling. Compared with kits from other suppliers and with kits using standard RNase H- MMLV RT, reactions with qScript Ultra carried out at 60°C show improved detection. (**A**) amplification plot and (**B**) Cq comparisons are shown.