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qPCR

Leveraging automation in qPCR: exploring the challenges and benefits of using 1-step RT-qPCR master mixes with liquid handling instruments

Abstract

The indications for qPCR testing have increased dramatically in recent years. Several fields of study have benefited from the application of qPCR technology, such as molecular diagnostics, where the market is projected to continue to grow steadily due to the increasing prevalence of ailments such as infectious diseases and cancer [1]. At the same time, there is an immense need to support disease prevention and treatment for a rapidly growing older population [2]. With the advancement of robotics over the years, automation is becoming increasingly widespread among clinical, pharmaceutical, and biotechnology R&D laboratories. While automating reaction setup can increase the efficiency, reproducibility, and throughput of qPCR workflows, specific challenges (especially for 1-step RT-qPCR) such as benchtop stability, reagent handling, and reaction setup must be addressed to help ensure successful integration. This paper aims to provide insights into these challenges and thus enable researchers to make informed decisions about setting up automation in their qPCR testing workflows.

Introduction

Quantitative PCR (qPCR) is a widely used technique for applications such as gene expression analysis, nucleic acid quantification, and pathogen detection. One-step RT-qPCR combines reverse transcription and amplification steps into a single tube, thus reducing steps and workflow time. Its technical benefits include high sensitivity and specificity, reduced experimental variability, and reduced hands-on time. The integration of liquid handling instruments with 1-step RT-qPCR master mixes provides numerous advantages over manual processes. By automating the labor-intensive steps of RT-qPCR, such as pipetting, mixing, and plate setup, researchers can significantly reduce hands-on time and increase throughput. In addition, inconsistencies in manual pipetting can be minimized, leading to greater consistency and reliability of RT-qPCR results. However, it is important to understand and address the challenges associated with integrating automation into the RT-qPCR workflow, to help ensure optimal performance and reliable results. While using a liquid handler can prevent human error during manual setups, the selection of a RT-qPCR master mix that is compatible with automation is crucial for reliable and consistent results. Differences in reagent viscosity, composition, and stability can affect the accuracy, performance, and reproducibility of the reactions. Compatibility testing and optimization are necessary to help ensure seamless integration between the master mix and the liquid handling platform.

Benchtop stability

When fully assembled 1-step RT-qPCR reactions are left on the benchtop for extended periods of time, performance can deviate from reactions that are run immediately. PCR artifacts can form when primers are bound to a nonspecific locus at a temperature lower than the T_m and then extended by the polymerase. Indeed, experiments have shown that nonspecific products may form with increased on-bench time [2]. Since automated liquid handlers may process multiple plates and include time where assembled plates are left on the bench prior to running on the instrument, the stability of completely assembled RT-qPCR reactions is important for maintaining sensitivity, reproducibility, and accuracy. The Applied Biosystems™ TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix is a benchtop-stable, single-tube master mix optimized for rapid, sensitive, and reproducible detection of viral and bacterial pathogens. TaqPath DuraPlex 1-Step RT-qPCR Master Mix was developed to remain stable in assembled reactions for at least 8 hours and up to 24 hours at 24°C (Figures 1 and 2). Compared to other master mixes on the market, TaqPath DuraPlex 1-Step RT-qPCR Master Mix has improved benchtop stability, making it an ideal option for qPCR setup with an automated liquid handler.

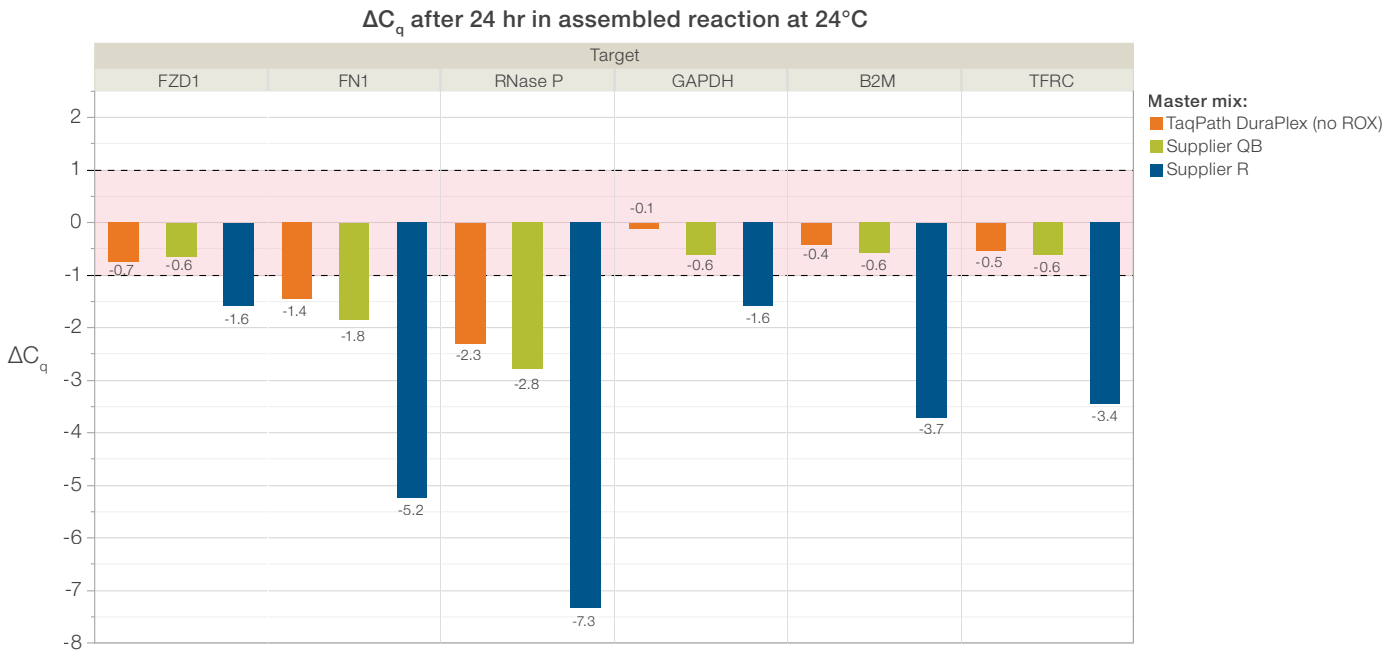

Figure 1. Benchtop stability of TaqPath DuraPlex 1-Step RT-qPCR Master Mix without ROX™ passive reference dye, and other suppliers' master mixes. A universal human reference RNA template was used at 100 ng in a reaction volume of 10 µL. A multiplex assay of 6 targets was used to assess benchtop stability of TaqPath DuraPlex 1-Step RT-qPCR Master Mix and master mixes from two other suppliers. Two sets of reactions were prepared: one set incubated for 24 hours at 24°C with the master mix, RNA, and 6-plex assay combined, and a set of freshly assembled reactions. The sets of reactions were run together on the same plate, using the recommended thermal cycling conditions for each master mix. ΔC_q was calculated as [C_q (freshly assembled reaction) – C_q (reaction incubated 24 hr)]. Targets and associated reporter dyes: *FZD1*, FAM™ dye; *FN1*, VIC™ dye; *RNase P*, ABY™ dye; *GAPDH*, JUN™ dye; *B2M*, cyanine 5 dye; *TFRC*, cyanine 5.5 dye.

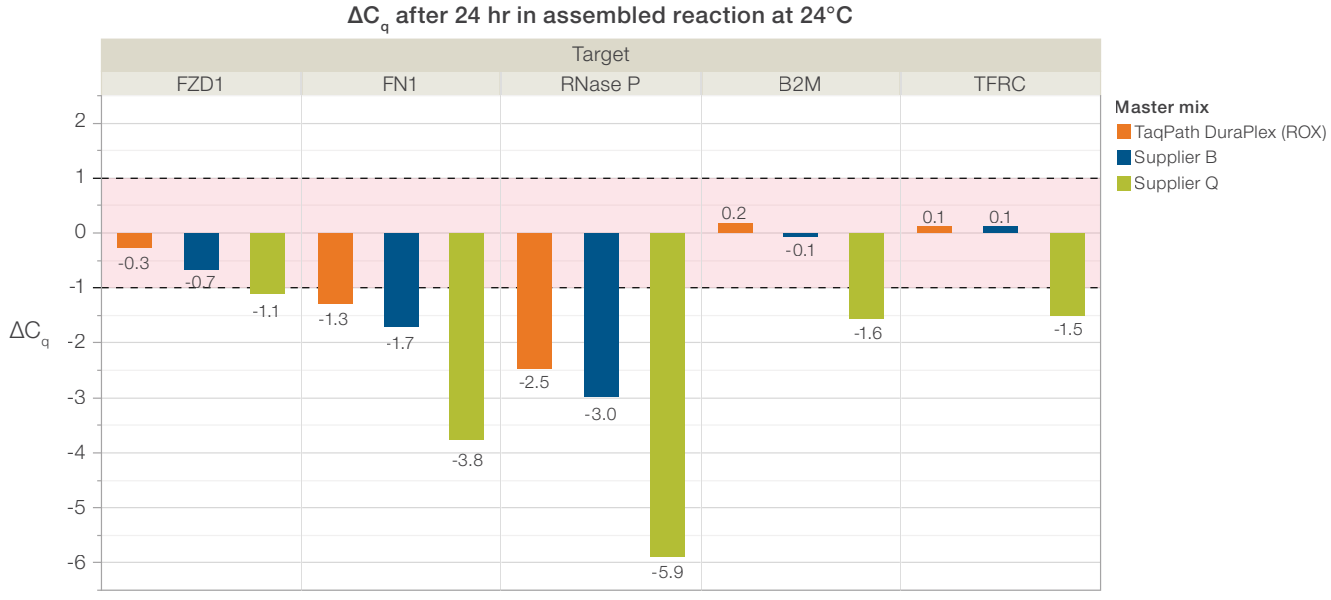

Figure 2. Benchtop stability of TaqPath DuraPlex 1-Step RT-qPCR Master Mix with ROX dye, and other suppliers' master mixes. A universal human reference RNA template was used at 100 ng in a reaction volume of 10 µL. A multiplex assay of 5 targets was used to assess benchtop stability of TaqPath DuraPlex 1-Step RT-qPCR Master Mix and master mixes from two other suppliers. Two sets of reactions were prepared: one set incubated for 24 hours at 24°C with the master mix, RNA, and 5-plex assay combined, and a set of freshly assembled reactions. The sets of reactions were run together on the same plate, using the recommended thermal cycling conditions for each master mix kit. ΔC_q was calculated as [C_q (freshly assembled reaction) – C_q (reaction incubated 24 hr)]. Targets and associated reporter dyes: *FZD1*, FAM dye; *FN1*, VIC dye; *RNase P*, ABY dye; *B2M*, cyanine 5 dye; *TFRC*, cyanine 5.5 dye.

Handling and setup

For successful integration with a liquid handler instrument, it is desirable to select a master mix that is easy to handle, robust, and stable for extended periods of time. Although typically providing greater sensitivity, master mixes of higher concentrations may result in higher viscosity. A highly viscous master mix can be difficult to pipette and result in insufficiently mixed reactions in both manual and automated processes. Viscosity can affect ease of handling during qPCR setup, and often produces inconsistent results between replicates in the same run and between experiments. Another potential undesirable characteristic of master mixes is foaming, which can cause insufficient reaction mixing during setup and irregular amplification curves. While a visual check can occasionally mitigate these problems in manual workflows, visual inspection is typically not possible with hands-off automated workflows. TaqPath DuraPlex 1-Step RT-qPCR Master Mix is offered at a 4X concentration to allow higher sample input in a single reaction, therefore helping to maximize sensitivity. Additionally, the master mix is formulated to allow easier handling and less foaming (Figure 3). Its viscosity is measured at 4.69 mPa·s, which is significantly lower than that of a similar on-market master mix tested, which measured at nearly 30% higher viscosity.

Repeated freeze-thaw cycles can also impact the stability and performance of 1-step RT-qPCR master mixes. If a master mix freezes during storage, aliquotting and storing it in smaller volumes is recommended, to minimize the potential impact of freeze-thaws on performance. Although preparing aliquots ahead of time helps to alleviate this issue, it increases time needed and reduces efficiency in the overall workflow. TaqPath DuraPlex 1-Step RT-qPCR Master Mix does not freeze at the recommended storage condition of –20°C and therefore does not freeze between uses, eliminating the need to prepare aliquots ahead of time. When tested in a scenario where the master mix vial is repeatedly removed from –20°C storage, exposed to light and ambient temperature for 1 hr, and returned to storage a total cycle of 20 times, the master mix shows no change in performance. In addition, when exposed to temperatures much lower than the recommended –20°C and freezing occurs, the product has demonstrated robust performance of up to 8 freeze-thaw cycles as shown in Figure 4 (with or without ROX dye).

A

B

Figure 3. Comparison of foaming between 1-step RT-qPCR master mixes. Different master mixes were assessed for bubble formation after assembly into reaction mixes. Mixing was performed by pipetting up and down 10 times to mimic mixing on liquid handlers. Reaction components are shown **(A)** before mixing and **(B)** after mixing. TaqPath DuraPlex 1-Step RT-qPCR Mix, on the far left, has less foaming after mixing than four other on-market 1-step RT-qPCR master mixes.

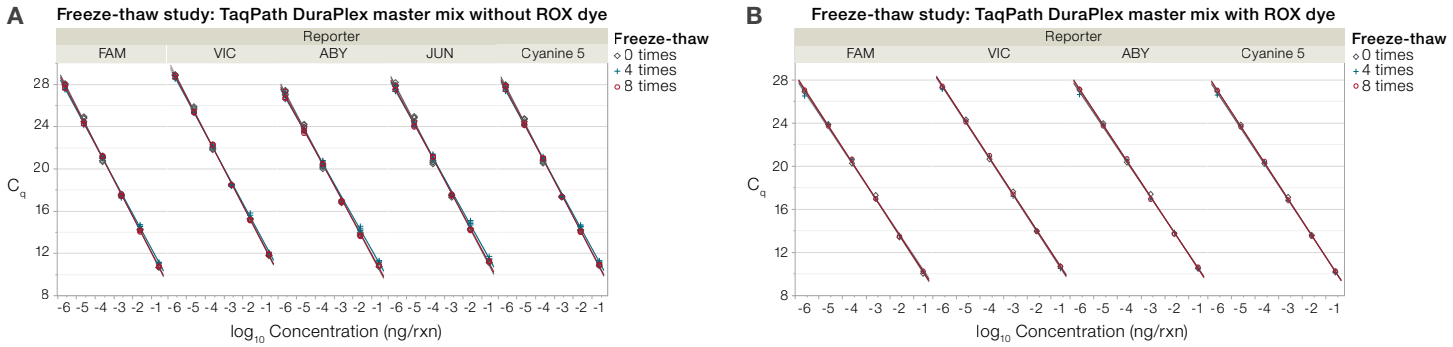

Figure 4. Performance of TaqPath DuraPlex 1-Step RT-qPCR Master Mix after freeze-thaw cycles. The master mix was placed at a temperature much lower than –20°C to allow freezing to occur. Once completely frozen, the reagent was placed at ambient temperature and allowed to thaw. Each freeze and thaw constituted 1 cycle. **(A)** TaqPath DuraPlex 1-Step RT-qPCR Master Mix without ROX dye shows similar performance across 6 serial dilutions in a 5-plex assay after multiple freeze-thaw cycles. **(B)** TaqPath DuraPlex 1-Step RT-qPCR Master Mix with ROX dye shows similar performance across 6 dilutions in a 4-plex assay after multiple freeze-thaw cycles. RNA input of 0.25 ng/rxn at dilution 1, and each subsequent input diluted 10-fold, were tested in both experiments. C_q values are plotted against RNA input to generate a linear regression for each condition by target. The R^2 value for all conditions tested with and without ROX dye is 1.00. qPCR dynamic range and efficiency are similar for 0, 4, and 8 freeze-thaw cycles.

Reagent stability

It is important to follow manufacturers' guidelines for proper storage to maintain the stability and performance of RT-qPCR master mixes. One-step RT-qPCR master mixes typically require specific storage conditions, including temperature below 0°C and protection from light. TaqPath DuraPlex 1-Step RT-qPCR Master Mix has a shelf life of 2 years from the date of manufacture when stored at –20°C. This robustness is ideal for liquid handler setups where the reagent will be exposed to ambient temperatures and light for extended periods of time. Ideally, the master mix of choice should be stable at 4°C or even at ambient temperature for some time frame to allow flexibility in handling and facilitate accurate detection.

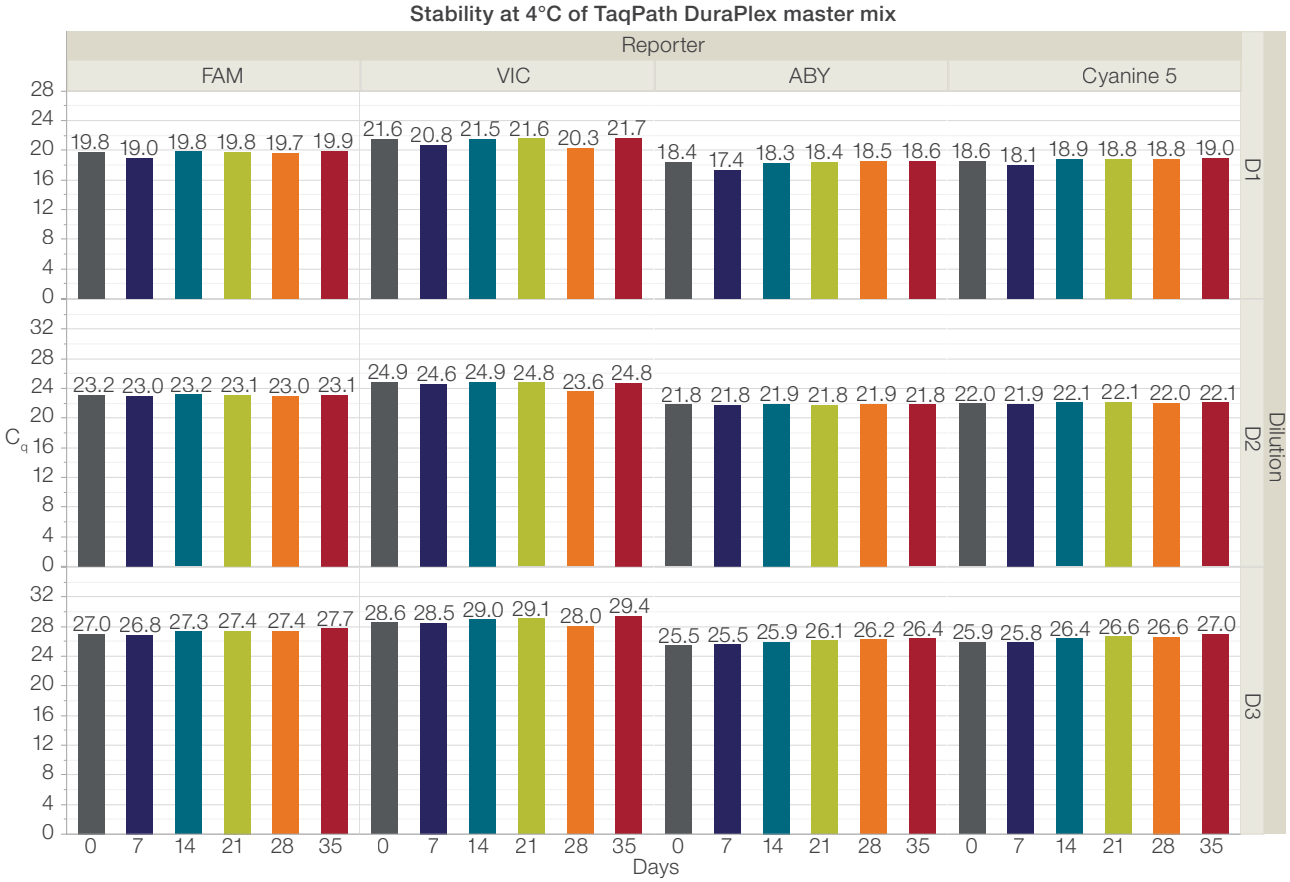

Figure 5. Stability of TaqPath DuraPlex 1-Step RT-qPCR Master Mix with ROX dye when stored at 4°C. The C_q values of reactions made with the master mix are plotted against days stored at 4°C. RNA input at 3 concentrations was tested with 4 targets in a single reaction. Performance of TaqPath DuraPlex 1-Step RT-qPCR Master Mix remains stable up to 35 days when stored at 4°C. The 4 reporter dyes were associated with different target regions on the input RNA.

To assess the stability when stored at temperatures higher than the recommended –20°C, TaqPath DuraPlex 1-Step RT-qPCR Master Mix was tested at the beginning of the study, and then stored at 4°C for a specific amount of time until it was tested for performance with a 4-plex assay. Compared to the master mix that was stored at –20°C (0 days), the master mix that was stored at 4°C for 35 days showed no difference in performance (Figure 5). Though not recommended, the master mix was further challenged by storage at 24°C or 34°C for 1 week and then tested against a control master mix stored at –20°C. Both master mixes stored at elevated temperatures remained similar in performance to the –20°C control master mix (Figure 6). Thus, for the duration of a typical workflow, researchers can minimize the chance of inaccurate or inconsistent results caused by reagent degradation. Altogether, a master mix with increased thermal stability typically offers greater compatibility with automated platforms and can help improve efficiency, accuracy, and consistency of testing.

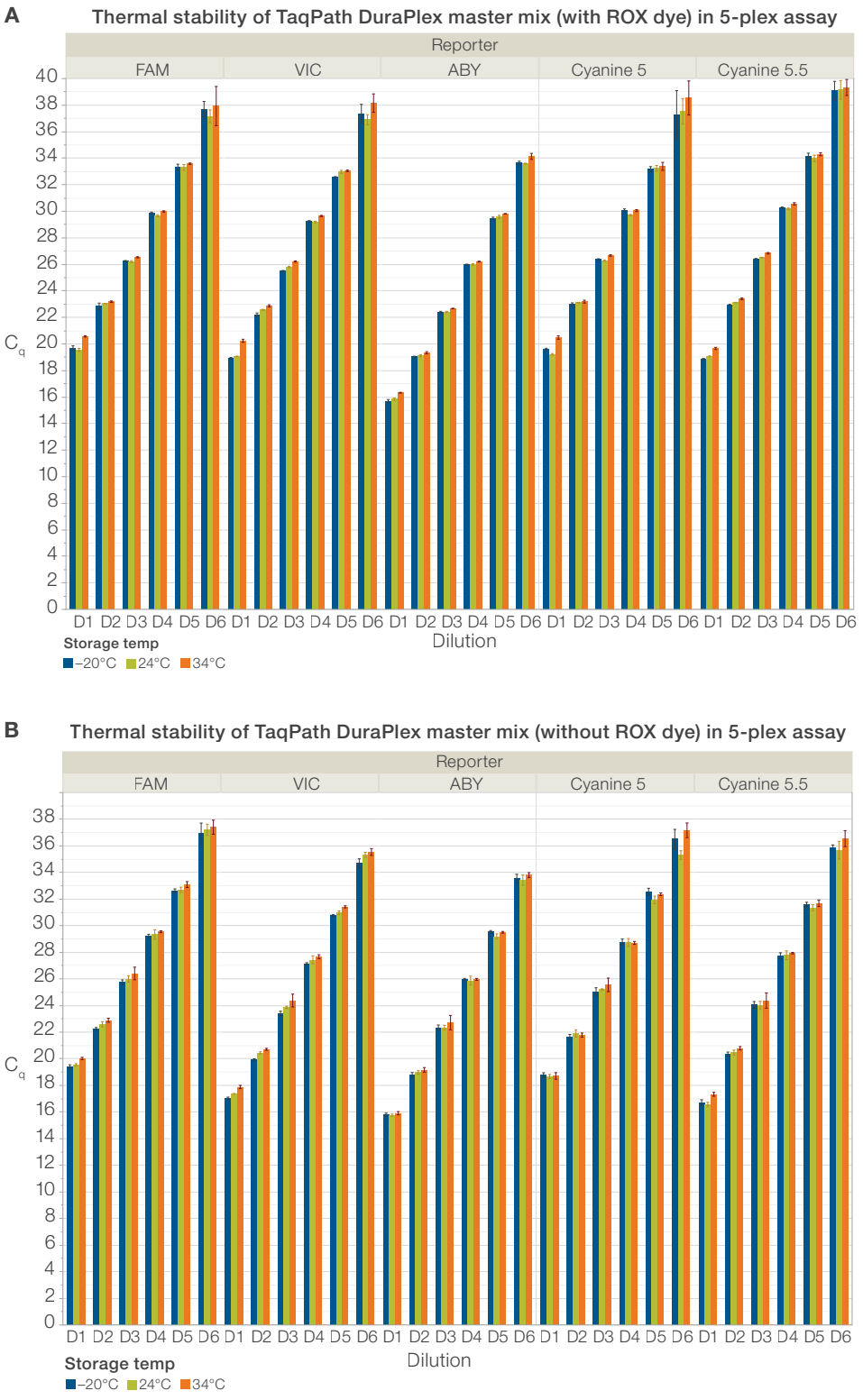

Figure 6. Thermal stability of TaqPath DuraPlex 1-Step RT-qPCR Master Mix at 24°C and 34°C. (A) TaqPath DuraPlex 1-Step RT-qPCR Master Mix with ROX dye shows no significant C_q changes after 1 week of storage at 24°C and 34°C compared to the –20°C control. **(B)** TaqPath DuraPlex 1-Step RT-qPCR Master Mix without ROX dye also performs similarly to the –20°C control after 1 week of storage at 24°C and 34°C. In both experiments, 5-plex human gene expression targets were tested: *ABCC1*, FAM dye; *APOE*, VIC dye; *CD44*, ABY dye; *ESR1*, cyanine 5 dye; *TXNDC1*, cyanine 5.5 dye.

Liquid handler compatibility testing of TaqPath DuraPlex 1-Step RT-qPCR Master Mix

Automation workflows in qPCR often require master mix and fully assembled reactions to be left on the benchtop for extended periods of time, which can result in changes in C_q. Furthermore, master mixes that are highly viscous often require optimization to work well with a liquid handler. To understand how TaqPath DuraPlex 1-Step RT-qPCR Master Mix performs with a liquid handler, the Biomek™ i7 Automated Workstation was used to set up a plate of complete RT-qPCR reactions that were run right away on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System. A 5-point dilution series of *in vitro* transcribed RNA was used with a 4-plex assay for the detection of SARS-CoV-2 targets. Using the liquid handler, TaqPath DuraPlex 1-Step RT-qPCR Master Mix was mixed with water and a 4-plex assay to make a bulk premix. Then, the premix was plated into an intermediate plate and transferred onto a final reaction plate, followed by addition of template RNA. The Biomek i7 workstation was able to set up 9 replicates for each of the dilution points with high consistency (Figure 7), with no special instructions for liquid class.

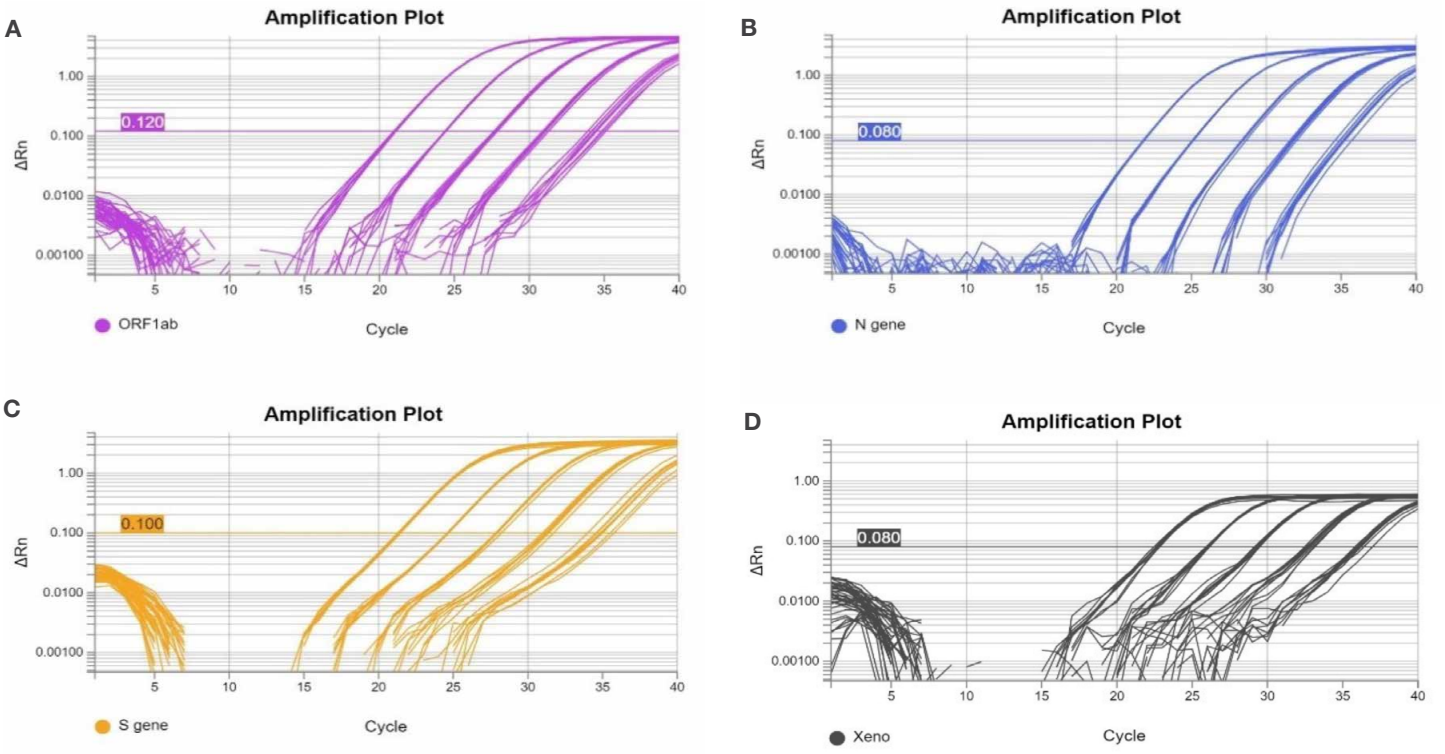

Figure 7. Compatibility of TaqPath DuraPlex 1-Step RT-qPCR Master Mix (with ROX dye) with a liquid handler setup. The Biomek i7 workstation was used to perform plate setup of a 4-plex assay to detect SARS-CoV-2 target sequences and Xeno RNA. All 9 replicates for each of the 5 dilutions were highly consistent. **(A)** *ORF1ab*, FAM reporter; **(B)** *N* gene, VIC reporter; **(C)** *S* gene, ABY reporter; **(D)** Xeno RNA, cyanine 5 dye.

To further investigate whether TaqPath DuraPlex 1-Step RT-qPCR Master Mix is compatible with liquid handling, an aliquot of the master mix was left exposed to light and ambient temperatures on the instrument deck for 24 hours. Then the Biomek i7 workstation was used to set up a plate of RT-qPCR reactions with this master mix and a 4-plex assay. This plate with fully assembled reactions was left on the bench for 8 hr prior to running on the QuantStudio 5 instrument. Compared to a plate that was run immediately, the plate with the assembled reactions left on the bench for 8 hr (and made with master mix that had been at ambient temperature for 24 hr prior to reaction assembly) showed no difference in performance (Figure 8). This experiment demonstrates a scenario where an automated process for RT-qPCR setup is successful due to selecting the right reagents, and thus enabling researchers to reduce human errors, minimize run-to-run variability, and increase throughput and overall efficiency.

Conclusion

The integration of 1-step RT-qPCR master mixes with liquid handling instruments offers substantial benefits, including time efficiency, accuracy, reproducibility, throughput, and error reduction. However, researchers must be aware of issues related to benchtop stability, storage conditions, handling, and workflow setup to help ensure reliable and robust results. TaqPath DuraPlex 1-Step RT-qPCR Master Mix is compatible with liquid handling instruments because of its ease of handling, robustness, and stability, all of which have been demonstrated in a realistic scenario with a common liquid handling platform. By selecting a compatible master mix and implementing appropriate protocols, researchers can harness the full potential of automation in 1-step RT-qPCR, supporting the advancement of scientific discoveries and applications in various fields.

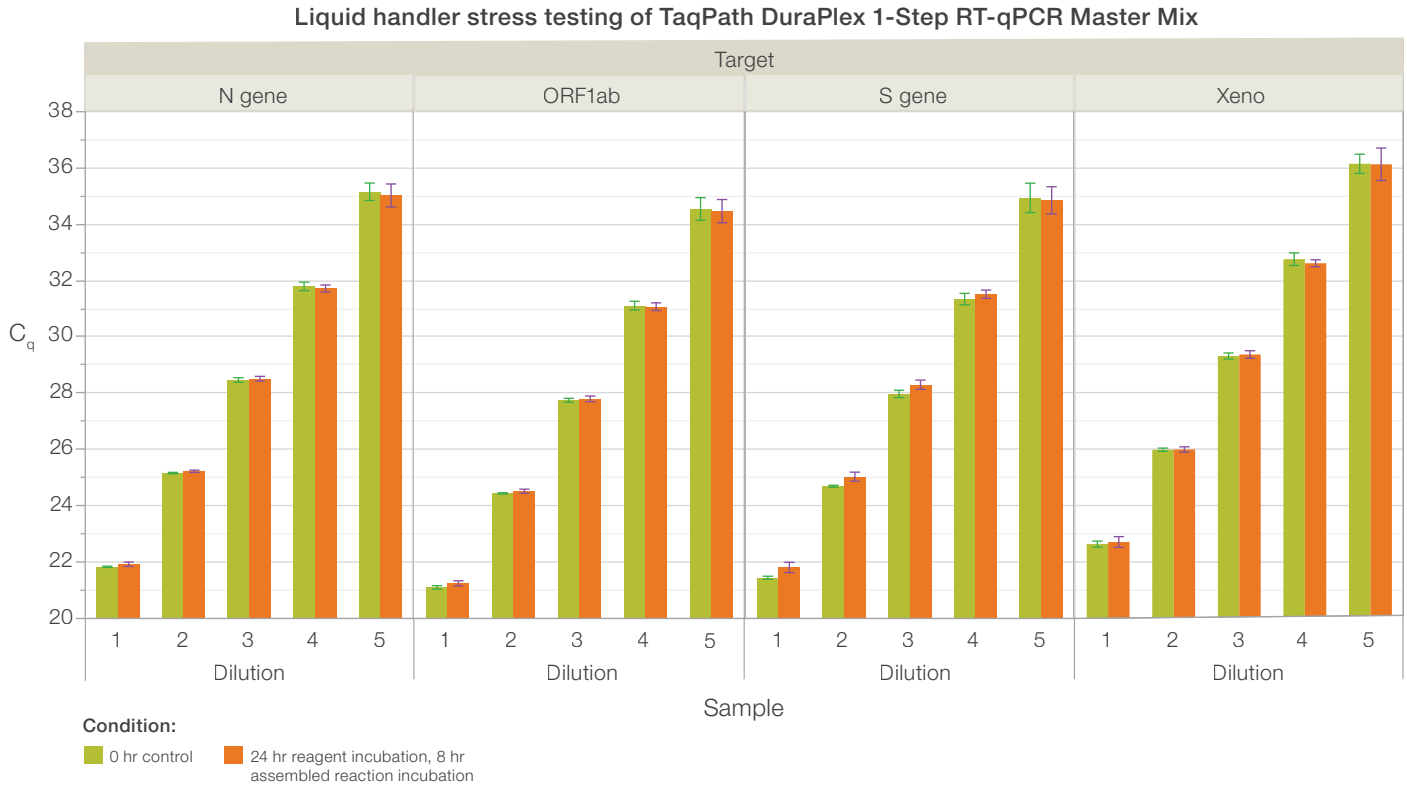

Figure 8. Stress testing of TaqPath DuraPlex 1-Step RT-qPCR Master Mix (with ROX dye) with a liquid handler setup. The master mix showed no change in C_q across 5 dilution points in a 4-plex assay after the reagent was exposed to ambient temperatures and light for 24 hr, and then used to assemble reactions that were left on the bench for 8 hr at ambient temperature before running on the QuantStudio 5 instrument. Target and associated reporter dyes: *ORF1ab*, FAM dye; *N* gene, VIC dye; *S* gene, ABY dye; Xeno RNA, cyanine 5 dye.

Ordering information

Product	Quantity	Cat. No.
TaqPath DuraPlex 1-Step RT-qPCR Master Mix	0.5 mL	A58666
	5 x 1 mL	A58667
	10 mL	A58668
TaqPath DuraPlex 1-Step RT-qPCR Master Mix (No ROX)	0.5 mL	A58669
	5 x 1 mL	A58670
	10 mL	A58671

References

1. Rathor RS (2023) Molecular diagnostics market: global forecast to 2028. Markets and Markets. **realtimeinsights.northernlight.com/document.php?docid=TM20230710050000020&datasource=TFMRASAP&trans=view&caller=all_results_widget&context=headline_card**
2. United Nations Department of Economic and Social Affairs. World populating ageing 2020. **un.org/development/desa/pd/sites/www.un.org.development.desa.pd/files/undesa_pd-2020_world_population_ageing_highlights.pdf**
3. Ruiz-Villalba A et al. (2017) Amplification of nonspecific products in quantitative polymerase chain reactions (qPCR). *Biomol Detect Quantif* 14:7–18. **ncbi.nlm.nih.gov/pmc/articles/PMC5727009/**

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