Lyophilization

Switch easily from liquid to lyophilized format using TaqMan Lyo-Ready master mixes with excipient

Highlights

- The growing demand for sustainable solutions leads to the development of lyophilized molecular diagnostic assays.
- Lyophilized assays can be stored and transported at ambient temperature, have a longer shelf life, and provide more room for samples.
- TaqMan Lyo-Ready 2.5X master mixes with excipient help facilitate seamless transition from conventional liquid to lyophilized versions of qPCR assays.

Introduction

As switching to more sustainable options becomes more important, lyophilization is becoming a popular solution to handle temperature-sensitive materials. One of the greatest benefits of having a lyophilized assay is the ability to store and ship at ambient temperature, avoiding energy-consuming cold chain handling logistics and providing a longer shelf-life. An additional desirable feature of a lyophilized reagent is that it allows a larger fraction of the final reaction volume to be allocated to the volume of the sample—this is particularly valuable when the input sample is very diluted.

However, besides the benefits mentioned above, lyophilization comes with certain challenges; the most significant being the lengthy and reagent-specific optimization process. It takes substantial time, effort, and knowledge to develop an optimal excipient that can protect enzymes during lyophilization and allow fast and seamless resuspension or dissolution of the lyophilized material when liquid sample is added. Another time-consuming step is the development of an optimal lyophilization-compatible master mix that can produce consistent results before and after lyophilization.

Thermo Fisher Scientific offers low-glycerol Applied Biosystems™ TaqMan™ Lyo-Ready master mixes with excipient—ready-to-use, one-tube solutions that just need to be mixed with primers and probes for direct lyophilization. Additionally, TaqMan Lyo-Ready master mixes with excipient come with comprehensive lyophilization guidelines to efficiently navigate the cumbersome protocol optimization from the start, substantially cutting the time to market. Once lyophilized, Lyo-Ready master mixes allow little to no loss of sensitivity or specificity and have been shown to reproducibly perform in high-multiplex reactions, even in the presence of common inhibitors.

Here we demonstrate the comparable performance of conventional glycerol-containing Applied Biosystems™ TaqMan™ and TaqPath™ master mixes and TaqMan Lyo-Ready master mixes with excipient, compared to master mixes from another supplier. Studies included testing the equivalence of linear dynamic ranges, multiplexing and limit of detection capabilities, and tolerance to inhibitors using a series of assays. The results demonstrate that customers can be confident in the effortless transition from conventional liquid to lyophilized assays using TaqMan Lyo-Ready 2.5X master mixes with excipient.

Conventional master mixes are incompatible with lyophilization

Lyophilization, or freeze-drying, is a process by which a reagent is stabilized by removing all the water content and restraining the activity of the enzymes in the reagent. The process requires the use of reagents that are compatible with lyophilization to ensure accurate performance after the process.

Conventional master mixes have glycerol, preservatives, and other lyophilization-incompatible reagents in their formulations, which makes them not suitable for lyophilization. TaqMan Lyo-Ready master mixes with excipient contain only lyophilization-compatible reagents and have been optimized to maintain performance standards after lyophilization to help ensure a smooth transition from conventional TaqMan and TaqPath master mixes to their lyophilized counterparts.

Transitioning from conventional to lyophilization-compatible qPCR master mixes

While transitioning from conventional to lyophilization-compatible master mixes, manufacturers are concerned about the change in performance of their assays. Main concerns include significant C_t value shifts, decrease in multiplexing capabilities, and detrimental effects on limit of detection, dynamic range, and inhibitor tolerance. A smooth transition with minimal changes to parameters, while ensuring our well-reputed brand manufacturing and quality controls, is the ideal scenario for most assay developers.

Here we demonstrate that TaqMan 2.5X Lyo-Ready master mixes with excipient retain the high-quality performance of conventional TaqMan and TaqPath master mixes, making it easier and more predictable to transition from liquid to lyophilized products.

Performance comparison: conventional vs. TaqMan Lyo-Ready master mixes with excipient

In Figure 1, the performance of TaqMan 2.5X Lyo-Ready master mixes with excipient was compared to conventional probe-based Applied Biosystems™ master mixes (Table 1), using the panel of assays described in Table 2.

For each assay, triplicate samples of 4 concentrations of a 10-fold template dilution series were quantified. TaqMan Fast Virus 1-Step Master Mix and TaqMan Fast Advanced Master Mix were used as references to determine performance changes as follows:

$\Delta C_t = Avg.Ct_{ref} - Ct_{test}$, with $|\Delta C_t| \leq 1$ considered not significant (dashed gray line);

$\%ddRn = (dRn_{test} - Avg.dRn_{ref}) / Avg.dRn_{ref}$, with $|\%ddRn| \leq 25\%$ considered not significant (dashed gray line); and

$\%dRaw.fl = (Raw.fl_{test} - Avg.Raw.fl_{ref}) / Avg.Raw.fl_{ref}$, with $|\%dRaw.fl| \leq 25\%$ considered not significant (dashed gray line).

All tested master mixes showed similar performance, with $|\Delta C_t| \leq 1$, as highlighted by dashed gray lines. While higher fluorescence was observed in some instances for TaqMan Lyo-Ready master mixes, most performance changes were minimal.

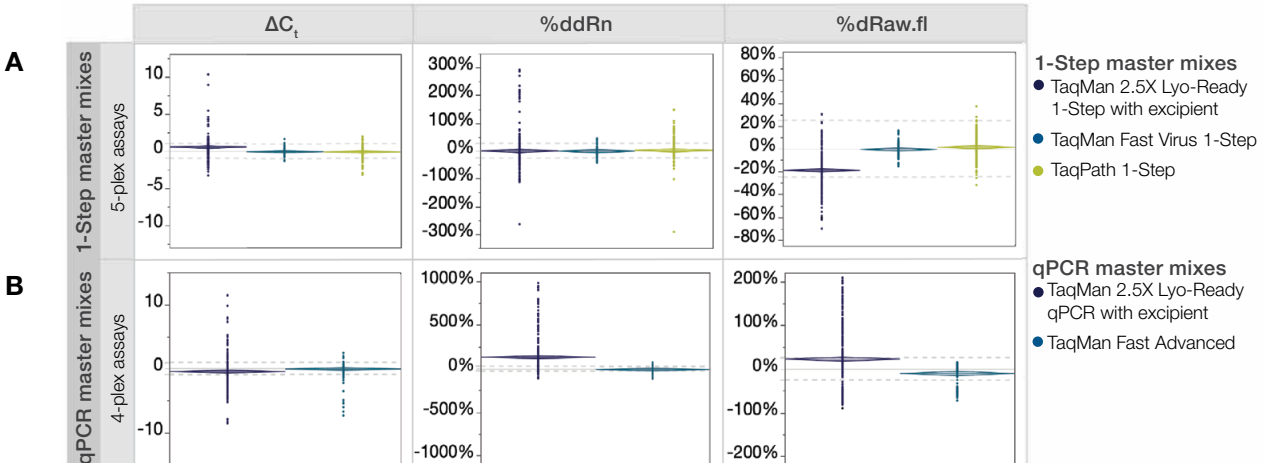


Figure 1. Performance of conventional and TaqMan Lyo-Ready master mixes. (A) Comparison of TaqMan 2.5X Lyo-Ready 1-Step Master Mix with excipient, TaqMan Fast Virus 1-Step Master Mix (reference), and TaqPath 1-Step Multiplex Master Mix using a panel of 5 different TaqMan Assays for detection of SARS-CoV-2, flu A virus, flu B virus, RSV, Zika virus, and MERS virus. **(B)** Comparison of TaqMan 2.5X Lyo-Ready qPCR Master Mix with excipient and TaqMan Fast Advanced Master Mix (reference) using a panel of 4 different TaqMan Assays (custom assays for the detection of *B. anthracis*, STEC, mastitis-causing pathogens, and genotyping).

Table 1. Composition highlights of master mixes used in this study. TaqMan 2.5X Lyo-Ready master mixes with excipient are highlighted in gray.

Type of master mix	Applied Biosystems™ master mix	Nucleic acid target	Composition highlights			
			Reverse transcriptase	UDG	dUTP	Excipient
1-Step	TaqMan™ 2.5X Lyo-Ready 1-Step Master Mix with excipient	RNA, DNA	+	–	–	+
	TaqMan™ Fast Virus 1-Step Master Mix, no ROX	RNA, DNA	+	–	–	–
	TaqPath™ 1-Step Multiplex Master Mix, no ROX	RNA, DNA	+	+	+	–
qPCR	TaqMan™ 2.5X Lyo-Ready qPCR Master Mix with excipient	DNA	–	–	–	+
	TaqPath™ BactoPure™ Microbial Detection Master Mix, no ROX	DNA	–	+	+	–
	TaqMan™ Fast Advanced Master Mix, no ROX	DNA	–	+	+	–

Table 2. List of assays and studies performed in this study.

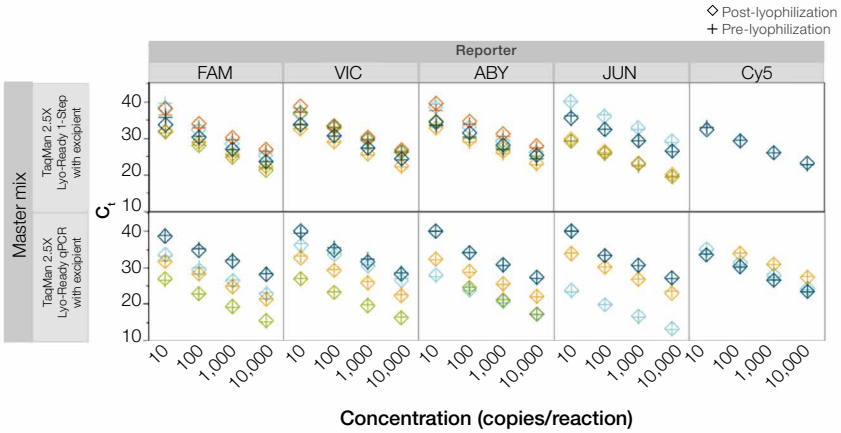
Type of master mix	Applied Biosystems™ assay	Target – reporter	Study
1-Step	TaqMan™ SARS-CoV-2, FluA, FluB RT-PCR Assay Kit with Xeno (5-plex)	• Flu A – FAM • SARS-CoV-2 – VIC • Flu B – ABY • MS2 – JUN • Xeno – Cy5	All studies
	TaqMan™ SARS-CoV-2, Flu A/B, RSV RT-PCR Assay Kit (Cat. No. A47702) with Xeno (5-plex)	• Flu A/B – FAM • SARS-CoV-2 – VIC • RSV – ABY • MS2 – JUN • Xeno – Cy5	Performance comparison: • Conventional vs. TaqMan Lyo-Ready master mixes with excipient • Pre- and post-lyophilization
	TaqMan™ SARS-CoV-2 with RNase P Assay 2.0 (4-plex)	• ORF1a – FAM • SARS-CoV-2 – VIC • ORF1b – ABY • RNase P – JUN	
	TaqMan™ Arbovirus Triplex Kit for Zika virus (4-plex)	• ZIKV – FAM • Den – VIC • CHIKV – ABY • PPIA – JUN	
	Custom assay for MERS virus detection (3-plex)	• ORF1a – FAM • UpE – VIC • TRFC – ABY	
qPCR	Custom assay for mastitis-causing pathogen detection (5-plex)	• *E. cloacae* – FAM • *S. marcescens* – VIC • *Enterococcus* – ABY • *P. aeruginosa* – JUN • Xeno – Cy5	All studies
	Custom assay for *Salmonella* and STEC detection (5-plex)	• Enterotoxic *E. coli*: – Wzy – FAM – STX1 – VIC – STX2 – ABY • *Salmonella* – JUN • Xeno – Cy5	Performance comparison: • Conventional vs. TaqMan Lyo-Ready master mixes with excipient • Pre- and post-lyophilization
	Custom assay for *Bacillus anthracis* detection (3-plex)	• pXO1-8 – FAM • pXO2-2 – VIC • TERT – ABY	
	Custom assay for genotyping (5-plex)	• CYP1B1 – FAM • FN1 – VIC • ABCC2 – JUN • RNase P – ABY • Xeno – Cy5	

Performance comparison: pre- and post-lyophilization

One of the main requirements for a lyophilization-compatible qPCR master mix is consistent performance before and after lyophilization. TaqMan 2.5X Lyo-Ready master mixes with excipient are designed to be incorporated directly into the lyophilization process and provide reproducible performance pre- and post-lyophilization (Figure 2). You can use a non-lyophilized version of TaqMan Lyo-Ready master mix with excipient to first optimize your assay. Once the assay is optimized, the lyophilized form of the master mix can then be incorporated, and any necessary minor changes to your assay design can be made. As lyophilization can be a long and challenging process, TaqMan 2.5X Lyo-Ready master mixes with excipient provide a convenient solution to lyophilized assay development.

Figure 2 shows the performance of the TaqMan 2.5X Lyo-Ready 1-Step Master Mix (upper panel) and TaqMan 2.5X Lyo-Ready qPCR Master Mix (lower panel) before and after lyophilization, evaluated using the multiplex assays described in Table 2. Both master mixes exhibited consistent performance pre- and post-lyophilization, which can enable quicker assay optimization and reduce the time to market.

A



B

Assay	ΔEfficiency	ΔR²
Zika	20.62%	0.015
MERS	12.49%	0.026
CoV / RNase P	5.61%	0.009
CoV / FluAB / RSV	6.70%	0.012
CoV / FluA / FluB	10.5%	0.004
B. anthracis	0.60%	0.005
STEC	1.85%	0.001
Genotyping	3.24%	0.002
Mastitis	2.21%	0.002

Figure 2. Performance of TaqMan Lyo-Ready master mixes with excipient pre- and post-lyophilization. (A) TaqMan Lyo-Ready 1-Step Master Mix (top) was evaluated using a panel of multiplex TaqMan Assays targeting SARS-CoV-2, flu A virus, flu B virus, RSV, Zika virus, and MERS virus; and TaqMan Lyo-Ready qPCR Master Mix (bottom) was evaluated with a panel of custom multiplex TaqMan Assays targeting genotyping targets, *B. anthracis*, STEC, and mastitis-causing pathogens. The overlapping crosses (pre-lyophilization) and diamonds (post-lyophilization) show consistent performance of the master mixes before and after lyophilization. **(B)** Average differences in PCR efficiency (ΔEfficiency) and R^2 (ΔR^2) for each assay before and after lyophilization. The low values indicate consistent performance of the master mixes before and after lyophilization.

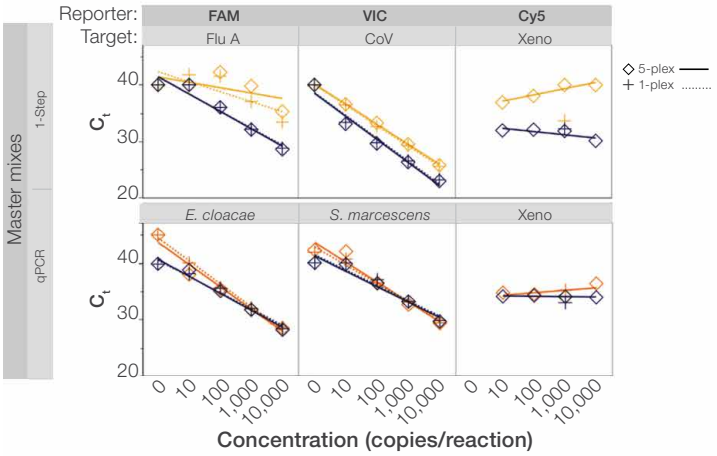
High multiplexing capabilities lead to more answers with fewer tests

It is extremely convenient to be able to detect multiple targets in a single sample while maintaining accuracy. Multiplexing can help save sample volume, handling time, and testing costs while achieving the same sensitivity and precision as for single-plex reactions. Another important aspect of multiplexing is the use of a master mix that is insensitive to competitive inhibition, meaning that higher copy number targets should not affect quantification of lower copy number targets.

In this study, high multiplexing capabilities of TaqMan Lyo-Ready master mixes with excipient were tested and compared to master mixes from supplier M using 5-plex TaqMan Assays shown in Table 2.

Templates from the 4 microbial targets were run in quadruplicate at concentrations of 10, 100, 1,000, and 10,000 copies per reaction. Additionally, a fixed concentration of Xeno template (10,000 copies/reaction) was used as a control for competitive inhibition (Figure 3). The results demonstrate the exceptional multiplexing capabilities of TaqMan 2.5X Lyo-Ready master mixes with excipient for 5-plex reactions, in comparison to supplier M's master mixes that struggled with competitive inhibition, as shown by the higher slope values for Xeno IPC. In addition, TaqMan Lyo-Ready master mixes with excipient (Figure 3A) showed little to no variation when comparing single-plex to multiplex reactions for all three targets shown (Figure 3B).

A



B

Master mix	Mean |1-plex – 5-plex| ΔEfficiency	ΔR²	ΔSlope$_{Xeno}$
TaqMan 2.5X Lyo-Ready 1-Step with excipient	1.80%	0.001	0.58
Supplier M	24.55% M	0.108 M	1.12
TaqMan 2.5X Lyo-Ready qPCR with excipient	5.65%	0.011	0.06
Supplier M	16.80% S	0.072 S	0.40

Figure 3. Comparison of high multiplexing capabilities of TaqMan Lyo-Ready master mixes with excipient to comparable master mixes from another supplier. (A) TaqMan Lyo-Ready 1-Step Master Mix and a comparable master mix from supplier M were evaluated using the 5-plex TaqMan SARS-CoV-2, FluA, FluB RT-PCR Assay Kit with Xeno IPC. TaqMan Lyo-Ready qPCR Master Mix and a comparable master mix from supplier M were evaluated using a custom 5-plex TaqMan Assay for mastitis-causing pathogen detection. Xeno template concentration was kept constant (10,000 copies/reaction) as a control for competitive inhibition. **(B)** Shown are the differences in PCR efficiency and R^2 values between 5-plex and 1-plex reactions (superscript "S" and "M" represent single-plex and multiplex reactions, respectively).

Specific target detection and quantification with wide dynamic range

For assay developers, using a reliable master mix with proven performance in accurately detecting molecular targets is a key to success. It is essential to be able to test a wide range of concentrations while maintaining an acceptable PCR efficiency (85–115%) and a good linear fit ($R^2 > 0.9$). Applied Biosystems master mixes enable specific target detection and quantification with wide dynamic ranges to facilitate reliable detection for assay development. The ideal scenario is to see insignificant shifts in C_t values when transitioning from a conventional to a lyophilization-compatible master mix.

The linear dynamic ranges of the various master mixes in Table 1 were compared using the 5-plex assays described in Table 2. Four replicates of a dilution series comprising at least 6 orders of magnitude were run for each master mix. As shown in Figure 4A, conventional TaqMan and TaqPath master mixes exhibited nearly equivalent C_t values as the TaqMan Lyo-Ready 1-Step Master Mix with excipient, providing confidence for a seamless transition from conventional liquid to lyophilized assays. Similarly, Figure 4B shows comparable C_t values among TaqMan Lyo-Ready qPCR Master Mix with excipient and conventional TaqMan and TaqPath master mixes. TaqMan Lyo-Ready qPCR Master Mix exhibited slightly lower C_t values than the conventional counterparts, but the differences were insignificant ($|\Delta C_t| < 1$).

All tested master mixes showed similar dynamic ranges with PCR efficiencies ranging between 87% and 103% and $R^2 > 0.996$, all within acceptable ranges. TaqMan Lyo-Ready master mixes exhibited higher ΔRn values than the other master mixes, indicating potentially increased sensitivity for some applications.

Reproducible low-level target detection

Another expectation of assay developers is highly reproducible, low-level detection from a wide variety of samples. Master mixes in Table 1 were compared to equivalent master mixes from another supplier for their ability to detect low-level targets. The same TaqMan Assays from previous experiments were used as comparison (Table 2). Four replicates containing 0, 5, 10, 100, or 1,000 copies of template per reaction were amplified in a 5-plex reaction. Two targets for each assay are shown as examples (Figure 5).

Compared to supplier M's master mix, all Applied Biosystems master mixes were able to discriminate between 0 and 5 copies of the template, even at a high multiplexing configuration. Supplier M's master mixes were unable to detect and differentiate at low template concentrations (<10 copies/reaction).

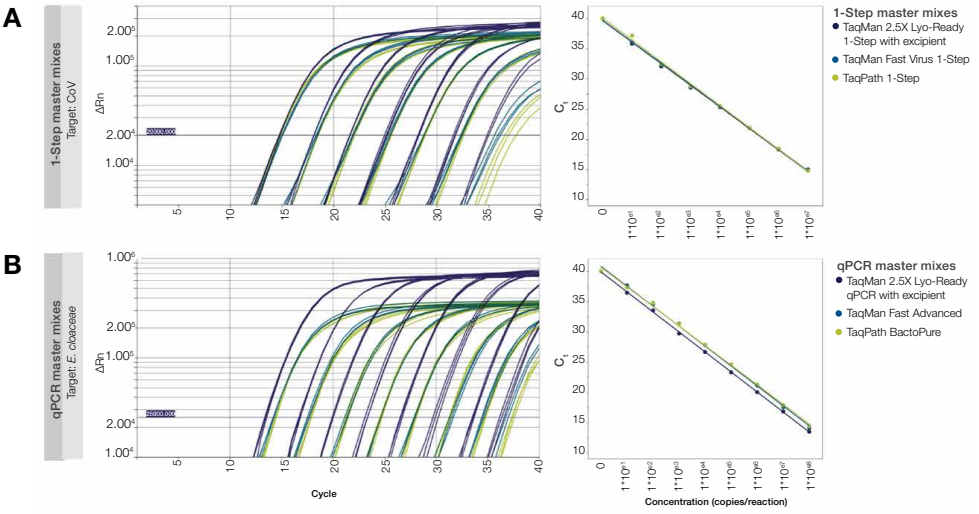


Figure 4. Linear dynamic ranges of Applied Biosystems master mixes. **(A)** Comparison of TaqMan Lyo-Ready 1-Step Master Mix, TaqMan Fast Virus 1-Step Master Mix, and TaqPath 1-Step Multiplex Master Mix. The master mixes were evaluated using a 5-plex TaqMan Assay for SARS-CoV-2, flu A virus, flu B virus, and Xeno IPC detection. **(B)** Comparison of TaqMan Lyo-Ready qPCR Master Mix, TaqPath BactoPure master mix, and TaqMan Fast Advanced Master Mix. The master mixes were evaluated using a custom 5-plex TaqMan Assay for mastitis-causing pathogen detection.

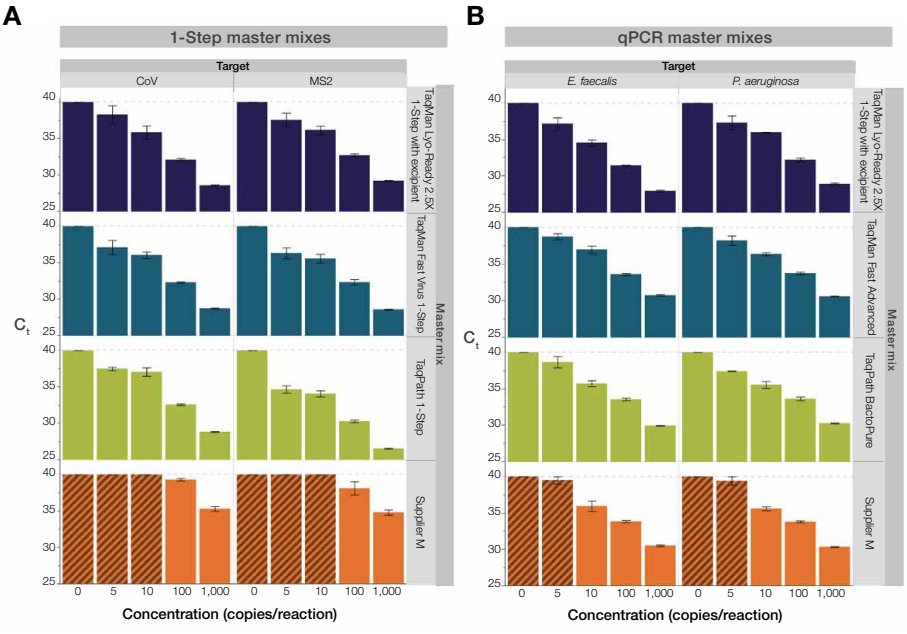


Figure 5. Limit of detection of Applied Biosystems master mixes compared to supplier M's master mixes. (A) Comparison of TaqMan Lyo-Ready 1-Step Master Mix, TaqMan Fast Virus 1-Step Master Mix, TaqPath 1-Step Multiplex Master Mix, and a comparable master mix from supplier M. **(B)** Comparison of TaqMan Lyo-Ready qPCR Master Mix, TaqMan Fast Advanced Master Mix, TaqPath BactoPure Microbial Detection Master Mix, and a comparable master mix from supplier M. TaqMan and TaqPath master mixes were able to successfully differentiate down to 5 copies of template per reaction, while supplier M's master mixes were not able to detect below 10 copies of template per reaction (Student's t-test, $p < 0.05$, 95% confidence; insignificant dilutions are shaded).

Reliable results for challenging samples

Another key feature that customers expect from master mixes is the ability to use the same master mix to analyze samples from various sources without being affected by inhibitors. Having one master mix with high inhibitor tolerance can help minimize supply costs, simplify the workflow, and shorten the time for data, as fewer sample preparation steps are required.

To demonstrate excellent inhibitor tolerance of the Applied Biosystems master mixes in Table 1, we performed two different experiments using the same TaqMan Assays as described in previous experiments (Table 2):

- In an initial test, relevant inhibitors that are usually found in molecular diagnostic (MDx) samples—IgG, NaCl, hematin, and heparin—were added at concentrations that are physiologically relevant for qPCR reactions [1-5].
- In a second test, crude lysates from samples—cell culture, saliva, and blood—were added at different ratios to the qPCR reactions.

1-Step master mixes (Table 1) were evaluated at lower inhibitor concentrations, as reverse transcriptase enzymes are more sensitive to inhibitors than DNA polymerases. Results for 1-Step master mixes and qPCR master mixes (Table 1) in comparison to equivalent master mixes from supplier M can be seen in Figures 6 and 7, respectively. All TaqMan and TaqPath master mixes showed higher inhibitor tolerance than supplier M master mixes, with low $|\Delta C_t|$ values across all targets between uninhibited and inhibited samples, even at concentrations higher than those expected of typical samples (dotted vertical lines in the figures) [1-5]. $|\Delta C_t|$ values of ≤1 between the uninhibited and inhibited samples are considered not significant (dashed horizontal gray line).

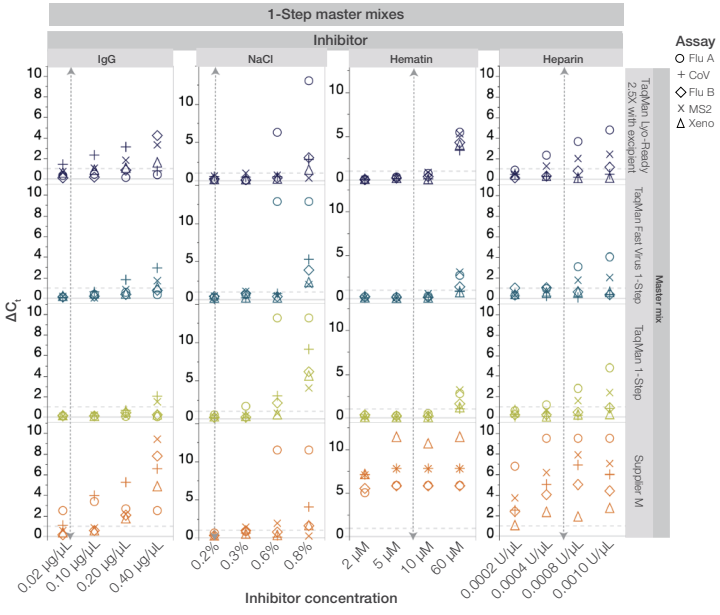


Figure 6. Compatibility of TaqMan Lyo-Ready 1-Step Master Mix with excipient to common inhibitors found in MDx samples. TaqMan Lyo-Ready 1-Step Master Mix, TaqMan Fast Virus 1-Step Master Mix, TaqPath 1-Step Multiplex Master Mix, and supplier M's master mix were evaluated for their compatibility with IgG, NaCl, hematin, and heparin using the same TaqMan Assays described previously. The dotted vertical lines indicate the maximum concentration of inhibitors found in diagnostic samples [1-5]. TaqMan and TaqPath master mixes consistently had the lowest $|\Delta C_t|$ values, even at inhibitor concentrations higher than the maximum concentration found in diagnostic samples.

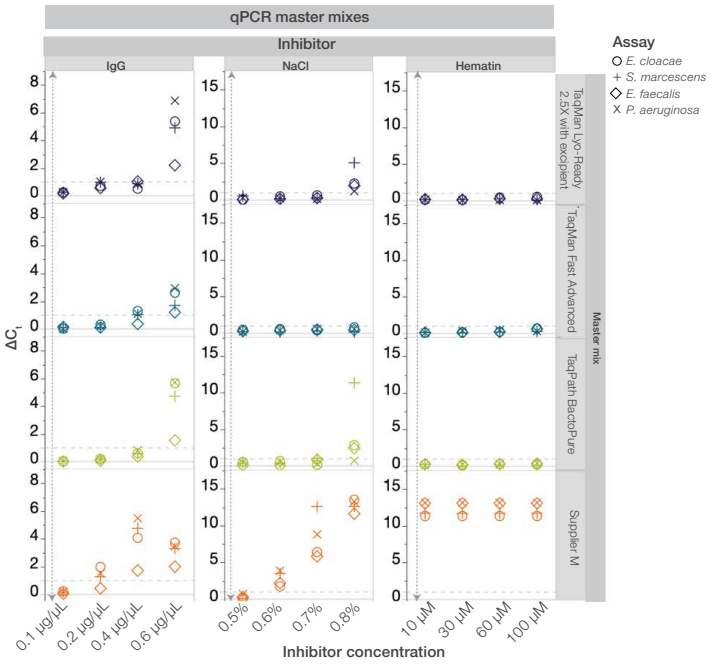


Figure 7. Compatibility of TaqMan Lyo-Ready qPCR Master Mix with excipient to common inhibitors found in MDx samples. TaqMan Lyo-Ready qPCR Master Mix, TaqPath BactoPure master mix, TaqMan Fast Advanced Master Mix, and supplier M's master mix were evaluated for their compatibility with IgG, NaCl, and hematin using the same TaqMan Assays described previously. The dotted vertical lines indicate the maximum concentration of inhibitors expected in diagnostic samples [1-5]. TaqMan and TaqPath master mixes consistently had the lowest $|\Delta C_t|$ values, even at inhibitor concentrations higher than the maximum concentration found in diagnostic samples. Supplier M's master mix showed greater variation in C_t values, with full inhibition even at the lowest hematin concentrations tested and below concentrations users may encounter in diagnostic samples.

Similar experiments were conducted using crude lysates from various sample types. Crude lysates were extracted using the Invitrogen™ Cells-to-C_T™ 1-Step TaqMan™ Kit (K562 cell culture samples), TaqMan SARS-CoV-2 Fast PCR Combo Kit (saliva samples), and Applied Biosystems™ DNA Extract All Reagents Kit (blood samples). The crude lysates were added to qPCR reactions at 4 different ratios of the reaction volume. The master mixes were evaluated using the same TaqMan Assays as in previous experiments (Table 2). The results for 1-Step master mixes and qPCR master mixes (Table 1) compared to equivalent master mixes from supplier M are shown in Figures 8A and 8B, respectively. All TaqMan and TaqPath master mixes showed compatibility with high percentages of crude lysates, as shown by the low $|\Delta C_t|$ values for uninhibited and inhibited samples.

When comparing the lyophilization-compatible master mixes, TaqMan 2.5X Lyo-Ready master mixes with excipient showed lower $|\Delta C_t|$ values for all targets for all concentrations of crude lysates. This was apparent at even high concentrations, including 80% concentration for saliva and cell culture samples for 1-Step and qPCR master mixes, respectively. This means that lyophilized products can be nearly resuspended in just crude lysate, without affecting performance. Supplier M's master mixes showed substantially lower compatibility with crude lysates, with higher $|\Delta C_t|$ values for most targets at lower concentrations, indicating higher susceptibility to the effects of PCR inhibitors.

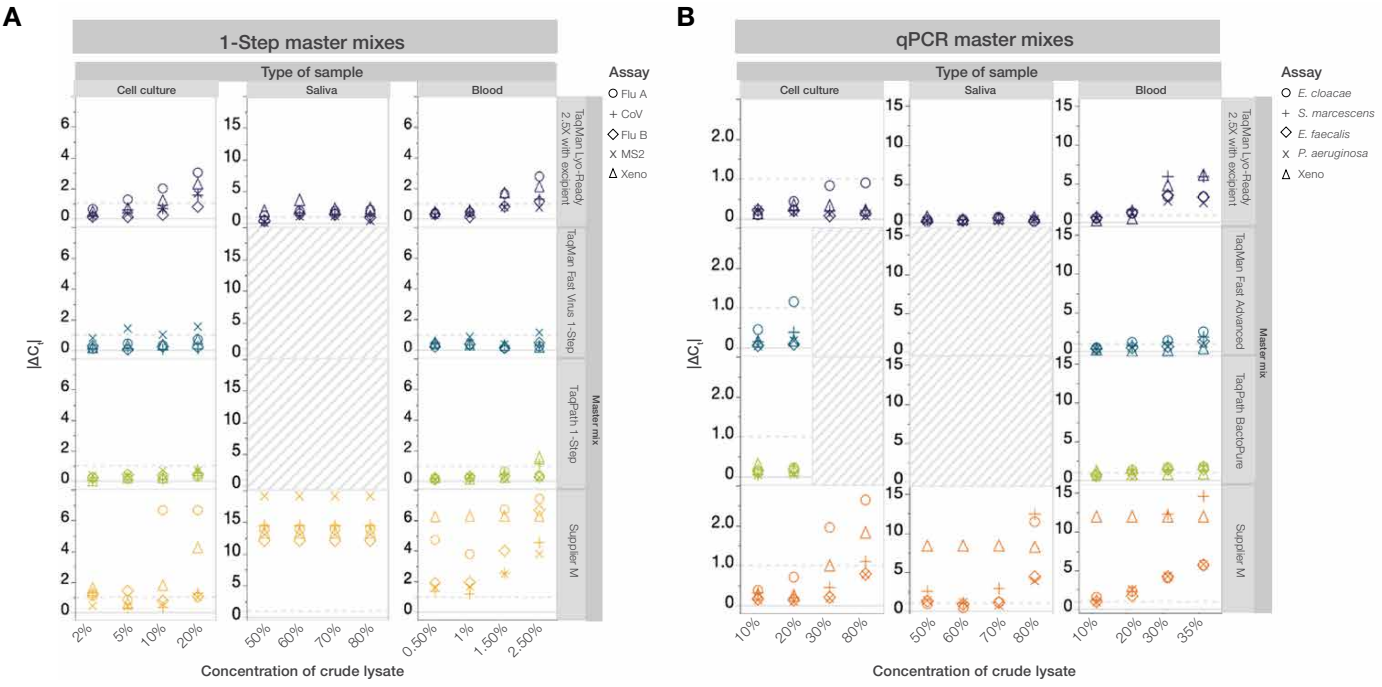


Figure 8. Compatibility of TaqMan Lyo-Ready master mixes to inhibitors present in crude lysates from cell culture, saliva, and blood samples. (A) Comparison of TaqMan Lyo-Ready 1-Step Master Mix with TaqMan Fast Virus 1-Step Master Mix, TaqPath 1-Step Multiplex Master Mix, and a comparable master mix from supplier M. **(B)** Comparison of TaqMan Lyo-Ready qPCR Master Mix with TaqPath BactoPure master mix, TaqMan Fast Advanced Master Mix, and a comparable master mix from supplier M.

Conclusion

Partnering with Thermo Fisher can help reduce the time and expenses associated with lyophilized qPCR assay development. TaqMan Lyo-Ready master mixes with excipient were developed using the knowledge gained from decades of experience in manufacturing qPCR products. Lyophilization-compatible master mixes were developed to help ensure consistent and reliable performance in a wide range of conditions: low copy numbers, high copy numbers, and multiplexing of inhibitor-rich samples. TaqMan Lyo-Ready master mixes with excipient are provided with comprehensive lyophilization guidelines to enable an easy transition from conventional to lyophilized master mixes.

References

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