Advancing Tools for the Development of Lyophilized qPCR Assays

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ABSTRACT

There is a need for simple, effective, and stable assays for the monitoring of viral outbreaks worldwide. One-step qPCR is a popular choice for RNA virus detection, due to its simplicity and lower risk of contamination. A drawback to one-step qPCR is that thermolabile components, such as reverse transcriptase enzyme, require cold chain shipment and storage. This is a hindrance to stockpiling material for quick response to outbreaks, as well as complicating shipping, especially for laboratories operating in areas where transportation logistics represent a significant challenge. Development of lyophilized assays is a potential solution to simplifying the workflow, storage and supply chain.
Most master mixes available on the market are not ideal for development of lyophilized assays, due to the presence of incompatible components. We have modified the high performing TaqMan® Fast Virus one-step qPCR master mix (Thermo Fisher Scientific PN 4444436) into a Lyo-ready one-step qPCR format. Here we demonstrate comparable performance, both pre and post lyophilization, as well as feasibility data for post-lyo stability at ambient storage. We then combined this formulation with a multiplex abrovirus (ZIKV) assay, and lyophilized directly in PCR tubes that can be shipped and stored at ambient temperature (for research use only, not for diagnostic use). The end user will only need to add purified nucleic acid.

INTRODUCTION

Lyophilization (freeze-drying) is accomplished through freezing of the product, then the removal of water via sublimation. Unlike conventional drying, the low temperatures prevent thermal degradation. Components that interfere with these processes, such as glycerol and volatile solvents, must be removed for successful lyophilization. 1-Step qPCR formulations are often composed vital components which contain these compounds (i.e. glycerol in enzyme buffers). It is a challenge to rebuild 1-Step qPCR formulations around lyo compatibility.

Here, a modification TaqMan® Fast Virus 1-Step master mix (Thermo Fisher Scientific PN4444436) was reformulated to be compatible with lyophilization, and tested for preservation of performance. We then combined the Lyo Ready 1-Step with our in-house processes to develop a TaqMan® Zika Virus Triplex Assay, lyophilized directly in a qPCR reaction well.

MATERIALS AND METHODS

The formulation for TaqMan® Fast Virus 1-Step master mix (Thermo Fisher Scientific PN4444436) was modified, removing incompatible components and incorporating glycerol free versions of the AmpliTaq® Fast DNA Polymerase and Thermostable MMLV Reverse Transcriptase (both from Thermo Fisher Scientific) to create a lyophilization compatible 1-Step qPCR master mix (Lyo-Ready 1-Step).

The Lyo-Ready 1-Step performance was compared to TaqMan® Fast Virus , using multiple TaqMan ®assays. The example shown is ACADVL (assay ID Hs00817723_g1, Thermo Fisher Scientific) across a log dilution series of human universal RNA (Agilent Technologies part# 740000). Testing was performed on a ViiA ™7 Real-Time PCR System, using the recommended cycling conditions for TaqMan® Fast Virus .

The stability of the Lyo-Ready 1-Step master mix was tested by amplifying across a dilution series XenoRNA ™ control with TaqMan® Gene Expression Assay (TaqMan® Cells-to-C_T™ Control Kit , Thermo Fisher Scientific part# 4386995). The formulation at was then stored at -15˚C to -25˚C for 1 year, and retested under the same conditions.

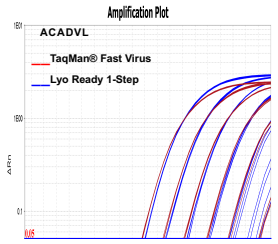
To demonstrate the feasibility of successful lyophilization, the Lyo-Ready 1-Step Master Mix was combined with a unique blend of excipients and aliquoted into MicroAmp™ Fast 8-Tube Strips (Thermo Fisher Scientific part# 4358293), at 25ul volumes. The material was lyophilized on an FTS Systems LyoStar™ II system, using an internally optimized lyophilization cycle. The qPCR performance of the lyophilized pellets was compared to the Lyo-Ready 1-Step Master Mix and TaqMan® Fast Virus , using a published assay sequence for MS2 phage RNA (MS2-TM2 [1] with VIC probe) and a dilution of 1.E+03 to 1.E+09 copies of MS2 RNA (US Biological part#R2033-18). Lyophilized 1-Step reactions, containing TaqMan® MS2 (MS2-TM3 [1] with FAM probe), in 8-well fast tube strips, were packaged in packaged moisture resistant pouches, with desiccant. The pouches were stored at 24˚C and tested at intervals, to demonstrate feasibility for ambient storage.

A Lyo-Ready 1-Step formulation was combined with a multiplex abrovirus assay, adapted from published assays [2-4] along with a PPIA endogenous control assay and Mustang Purple™ passive reference dye. This was developed into TaqMan ® Zika Virus Triplex Kit (Thermo Fisher Scientific , custom part# A31746* and A31747*) for research use.

*For Research use only..

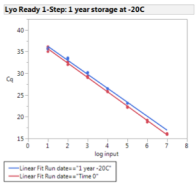
RESULTS

Figure 1. Amplification curve comparisons



Once modified to a Lyo-Ready master mix, it is critical to maintain qPCR performance. A representative example is shown in Figure 1: Lyo ready 1-Step (blue) and TaqMan® Fast Virus 1-Step master mix control (red), with a gene expression assay (ACADVL) across a dilution series of human universal RNA (0.001ng-100ng/25ul reaction). Of nine of ten assays tested, the Lyo Ready 1-Step master mix maintained both Cq (within ±1) and fluorescence (dRn), when compared to Fast Virus (data not shown).

Figure 2. Pre-Lyo Stability at -20C storage

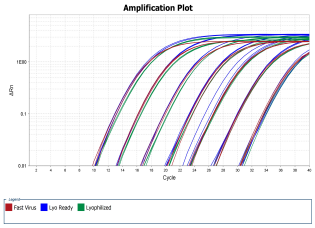


Prior to being put through a lyophilization process, the Lyo-Ready 1-Step mix must still be shipped and stored under frozen conditions. In order to confirm post modification stability under -15 to -25C storage conditions . The Lyo-Ready 1-Step was tested immediately after formulation, using XenoRNA™ TaqMan® Gene Expression Assay . The formulation was stored frozen for 1 year, then retested under the same conditions. In Figure 2 , Cqs are plotted against log concentration (copies of Xeno RNA /20ul reaction), while Table 1 compares the mean Cqs. The material stored for 1year demonstrates similar Cq performance to the time 0 test, as well as high PCR efficiency and r^2, indicating that it has maintained stability under these storage conditions.

Table 1. Comparison of Cq at 1 year -20˚C Storage

Copies /25ul rxn	Run 3/8/2016 Mean	Std Dev	Run 3/3/2017 Mean	Std Dev
10mill	16.03	0.14		
1mill	19.06	0.26		
100,000	22.27	0.12	23.15	0.04
10,000	25.77	0.12	26.47	0.07
1,000	29.18	0.23	30.15	0.08
100	32.41	0.37	33.44	0.15
10	35.61	0.58	35.73	0.15
NTC	40.00	0.00	38.13	0.23
slope	-3.30		-3.21	
%PCR eff	101.00		104.71	
R2	0.998		0.994	

Figure 3. Comparison of Pre-and Post lyophilization



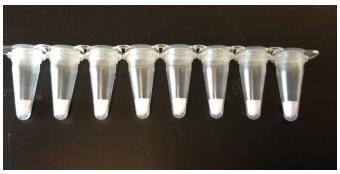
A Lyo-Ready 1-Step master mix was combined with excipients and lyophilized in 25ul volume pellets, using an internally optimized process. In Figure 3, the lyophilized reactions (green) are compared to the Lyo-Ready 1-Step mix (blue), as well as TaqMan™ Fast Virus 1-Step Master mix (red), across a dilution series of purified MS2 phage RNA. The overlapping amplification curves demonstrate retained qPCR performance in the lyophilized format.

Table 2. Lyophilized Stability after 1 year ambient storage

Copies MS2 RNA/25ul reaction	mean Cq	
	Time 0	1year @24˚C
1.E+06	21.48	21.46
1.E+04	31.98	31.77

Lyophilized 1-Step reactions were packaged and stored at 24˚C. The reactions were tested at intervals, using and input of 1million copies/25ul reaction (2 replicate wells), and 10,000copies/25ul reaction (4 replicate wells). In Table 2 , the Cq at 1 year storage are compared to those at time 0. The qPCR performance was highly conserved under ambient storage conditions.

Figure 4. Image of lyophilized reagents in MicroAmp™ Fast PCR tube-strip



lyophilized 1-Step master mix reactions in an 8-well MicroAmp™ Fast tube strip are shown in Figure 4. After lyophilization, reactions are sealed with a cap strip and packaged, with desiccant, in moisture resistant pouches for storage.

Figure 5. Lyophilized Reaction Workflow

The 1-Step qPCR workflow with the lyophilized format is simplified. Purified RNA is added directly to the reaction tube, minimizing handling time. Because all of the reaction components, including primers and probes, are stabilized in the pellet, the entire reaction volume can be comprised of the sample, maximizing target input.

Table 3. TaqMan™ Zika Virus Triplex Assay Targets

Filter	Dye	Target
1	FAM	Zika
2	VIC	Pan-dengue
3	ABY	Chikungunya
4	JUN	PPIA Cyclophilin (endogenous control)
5	MP	Passive reference

Table 4. TaqMan™ Zika Virus Triplex Kit cycling conditions

Step	Cycling condition	
Reverse Transcription	Hold	50˚C for 20min
Polymerase activation	Hold	95˚C for 2min
PCR amplification	40 cycles	95˚C for 15 sec, 60˚C for 1min

Figure 6. Positive control results using inactivated RNA viruses and *PPIA* endogenous control gene

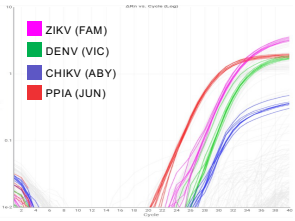


Figure 6 shows an example of real-time results, from 8 lyophilized reactions containing the using inactivated virus RNA (Vircell, Granada, Spain) for ZKIV, CHIKV, and DENV targets. All three amplicons and the *PPIA* control gene are amplified in a single 25ul reaction, using the cycling conditions described in Table 4. This application of lyophilized 1-Step reactions is now a custom product from Thermo Fisher Scientific A31746 and A31747 (for research use only).

Figure 7. Lyophilized Reactions Packaged in a Moisture Resistant Pouch

Figure 8. Lyophilized Reactions in a 96-well plate

CONCLUSIONS

Enhanced stability and shelf life, ambient shipping and storage, as well as greater workflow simplicity make lyophilized 1-Step qPCR assays ideal for rapid testing of viral RNA. Lyophilization processes vary greatly depending on many factors, including: formulation, volume, lyophilization vessel , and end use. Our goal was to develop a robust lyophilization compatible 1-Step qPCR master mix that is flexible enough to be inserted into lyophilized assay development with minimal optimization.

Here we have demonstrated a lyophilization compatible 1-step master mix that maintains high qPCR performance. We have demonstrated the achievability of inserting this Lyo ready mix into a freeze drying process, without detriment to function. These lyophilized reactions can be stored at ambient temperature, reducing the burden of cold storage.

We combined these tools to develop a custom lyophilized 1-Step multiplex assay for the study of RNA arboviruses, including Zika.

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TRADEMARKS

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