Challenge
Achieving comparable results with liquid and lyophilized One-Step RT-qPCR Master Mix in both singleplex and multiplex real-time PCR reactions.

Solution
The qTOWERiris ensures reproducible and comparable results regardless of well format, master mix type and multiplex capabilities.

Intended audience
General qPCR users who evaluate to use lyophilized master mix.

Comparing Liquid and Lyophilized One-Step RT-qPCR Master Mix Using qTOWERiris

Introduction

One-Step RT-qPCR master mix consists of all reagents ready-to-use for direct RNA analysis including cDNA synthesis for various applications. The entire reaction chain takes place in the same vessel, which simplifies the workflow and reduces the risk of contamination, minimizing pipetting errors and enhances throughput capabilities[1].

Lyophilization (freeze-drying) of qPCR reagents ensures long-term stability at higher temperatures and increased stability at room temperature, reducing the need for cold chain logistics and allowing for easier storage and transport[2]. This stability extends the shelf life of the reagents, ensuring they remain effective over longer periods. This reliability is crucial for obtaining reproducible and accurate qPCR results making them suitable for a wide range of applications, including multiplex assays.

This application note describes the compatibility of the liquid Luna® Probe One-Step RT-qPCR 4X Mix and the lyophilized LyoPrime Luna One-Step RT-qPCR Mix from New England Biolabs to the real-time qPCR thermal cycler qTOWERiris. We evaluated the compatibility based on key qPCR performance attributes such as reproducible measurements, PCR efficiencies and multiplex capabilities.

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Materials and Methods

Samples and reagents

- MVP Human Total RNA, Agilent Technologies
- Luna® Probe One-Step RT-qPCR 4X Mix (cat.: M3019S), New England Biolabs
- LyoPrime Luna® One-Step RT-qPCR Mix (cat.: L4001S), New England Biolabs
- GAPDH specific primers and probe for detection using Color Module 2
- RhesusD specific primers and probe for detection using Color Module 4
- Beta actin specific primers and probe for detection using Color Module 5
- 96-well PCR Plate (0.2 mL; low profile), full-skirted, white, 844-70038-S (Analytik Jena GmbH+Co. KG)
- 384-well PCR Plate, full-skirted, white, 844-70039-0 (Analytik Jena GmbH+Co. KG)
- Optical sealing foil, 844-70046-0 (Analytik Jena GmbH+Co. KG)

Instrumentation

- Real-time PCR thermal cycler qTOWERiris (Analytik Jena GmbH+Co. KG) including:
  - Color module 2, green (520 nm/560 nm) for YakimaYellow® dye
  - Color module 4, orange (580 nm/620 nm) for ROX™ dye
  - Color module 5, red (625 nm/670 nm) for Cy5® dye
- Real-time PCR thermal cycler qTOWERiris 384 (Analytik Jena GmbH+Co. KG) including:
  - Color module 2, green (520 nm/560 nm) for Yakima Yellow® dye
  - Color module 4, orange (580 nm/620 nm) for ROX™ dye
  - Color module 5, red (625 nm/670 nm) for Cy5® dye

qPCR settings

To evaluate the robustness for compatibility between qTOWERiris and the liquid and lyophilized RT-qPCR mix, three key parameters for qPCR were determined using the PCR cycler conditions shown in Table 1:

1. **Uniformity of Ct values across the PCR block**
   Replicate reactions were setup with pre-diluted total RNA (90 ng/µL), GAPDH specific primers and probe, which were added to liquid and lyophilized master mix. The two reaction types were transferred in 20 µL and 10 µL volume per well to one half of a 96-well and 384-well PCR plate each, respectively, to compare the measurement data within the same qPCR run. The same baseline correction and threshold values for Ct determination were used for both master mix types on one plate format to ensure similar calculation parameters.

2. **PCR efficiency of multiple gene targets**
   Standard curves were generated by generating five 1:10 dilutions of total RNA ranging from 90 ng/µL to 9 pg/µL. The different dilutions were added to liquid and lyophilized master mix and gene specific primers and probes (Table 2) on each half on a 96-well and 384-well plate. Linearity and efficiency of the amplification was determined by linear regression.

3. **Comparing the performance between singleplex and multiplex reactions**
   In addition to singleplex reactions of the three gene targets, a multiplex assay was conducted using the five RNA dilutions, gene specific primers and probes in a combined reaction for liquid and lyophilized master mix on each half of a 96-well PCR plate.

Method parameters

Table 1: One-Step RT-qPCR protocol

Step	Cycle	Profile	Temperature	Holding time	Ramp rates**
1	1	Carryover Prevention	25 °C	30 sec	8/4 °C/ sec
2	1	Reverse Transcription	55 °C	10 min	8/4 °C/ sec
3	1	Initial Denaturation	95 °C	1 min	8/4 °C/ sec
4	45	Denaturation	95 °C	10 sec	8/4 °C/ sec
		Annealing & Elongation*	60 °C	30 sec	5.5/2 °C/ sec

* Data acquisition: Color Module 2, Color Module 4, Color Module 5 using Gain 5
** Maximum ramp rates depend on the qTOWERiris model type (96/384)

Table 2: Overview of gene targets and probes

Gene targets	Amplicon length (bp)	GC content (%)	Fluorophore/Quencher
GAPDH	89	49	Yakima Yellow/BHQ-1
RhesusD	74	51	ROX/BHQ-2
ACTB	132	54	Cy5/BHQ-650

Results and Discussion

1. Uniformity of Ct values across the PCR block

Both master mix types were first evaluated in terms of reproducible and comparable results of Ct values and standard deviations across all replicate reactions in 96-well and 384-well format. Ct values were highly similar between liquid and lyophilized master mix measured in each of both plate formats (Figure 1). In the 96-well format, the standard deviation was < 0.5 with a coefficient of variation (CV) < 1% demonstrating a great performance of the master mix and qPCR cycler (Table 3). In the 384-well format, standard deviation was slightly above 0.5 with a CV of > 2%, which might be due to manual pipetting of smaller volumes of the reaction mix.

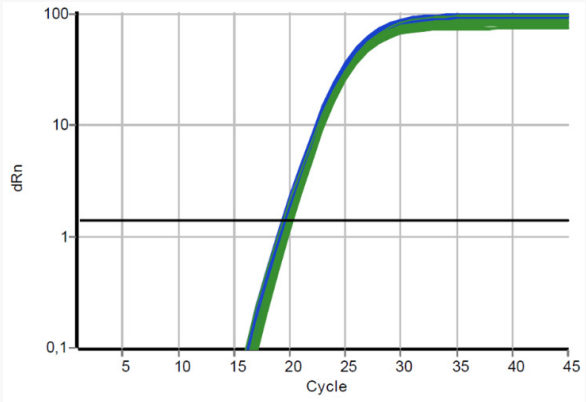

Figure 1a: Amplification of GAPDH gene using the liquid (green) Luna® Probe One-Step RT-qPCR 4X Mix and lyophilized (blue) LyoPrime Luna® One-Step RT-qPCR Mix from NEB in 96-well format of the qTOWERiris qPCR thermal cycler.

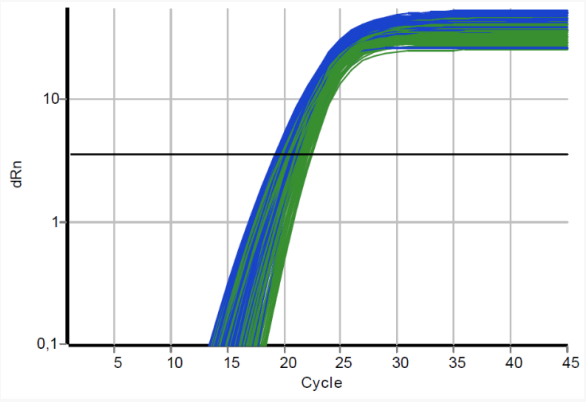

Figure 1b: Amplification of GAPDH gene using the liquid (green) Luna® Probe One-Step RT-qPCR 4X Mix and lyophilized (blue) LyoPrime Luna® One-Step RT-qPCR Mix from NEB in 384-well format of the qTOWERiris qPCR thermal cycler.

Table 3: Results of replicate amplifications of uniformity test between master mix types and plate formats

	96-well		384-well	
	Liquid	Lyophilized	Liquid	Lyophilized
Threshold	1.387		3.502	
Ct mean	19.65	20.03	20.20	20.94
Standard deviation	0.16	0.15	0.64	0.77
Coefficient of variation (%)	0.81	0.75	3.17	3.68

2. PCR efficiency of multiple gene targets

Standard dilution curves from three genes were generated for liquid and lyophilized master mix in both plate formats. Overall linearity coefficient R^2 of dilution curves was ≥ 0.98 for all genes regardless of the master mix types and plate formats showing adequate results using qTOWERiris (Figure 2).

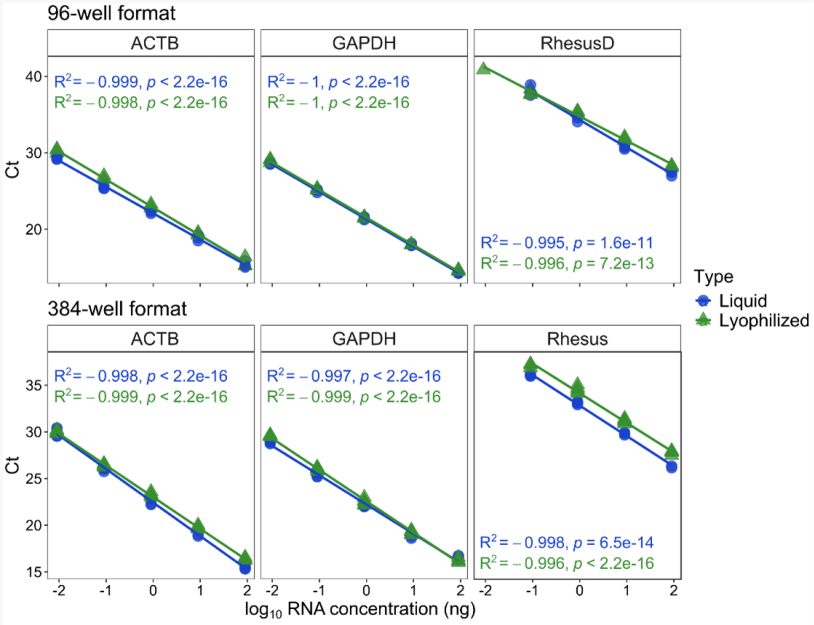

Figure 2: Comparison of standard curves of three genes in a 96-well and 384-well format for liquid and lyophilized One-Step RT-qPCR master mix. R^2 shows the linearity of Ct values against $\log_{10}$ RNA concentrations and *p* value represents significance of it.

3. Comparing performance between singleplex and multiplex reactions

The dilution curves of the three genes from singleplex reactions of the 96-well assay were compared to the multiplex assay of all genes in one reaction. Linearity showed high reproducibility across singleplex and multiplex reaction types for both master mix types (Figure 3).

Ct values and PCR efficiencies were finally compared between the master mix types (Figure 4). There were no significant differences of Ct values from all genes for both assay types between the liquid and lyophilized master mix. PCR efficiencies showed no significant difference between both master mix types except for the ATCB gene in the singleplex assay, which was not observed in the multiplex assay.

In conclusion, Luna® Probe One-Step RT-qPCR and LyoPrime Luna® One-Step RT-qPCR Mix demonstrated similar performance and results on the qTOWERiris. Tested performance attributes for qPCR showed excellent reproducibility, linearity and efficiency between the master mix types, which indicates outstanding compatibility between each other using the qTOWERiris qPCR thermal cycler.

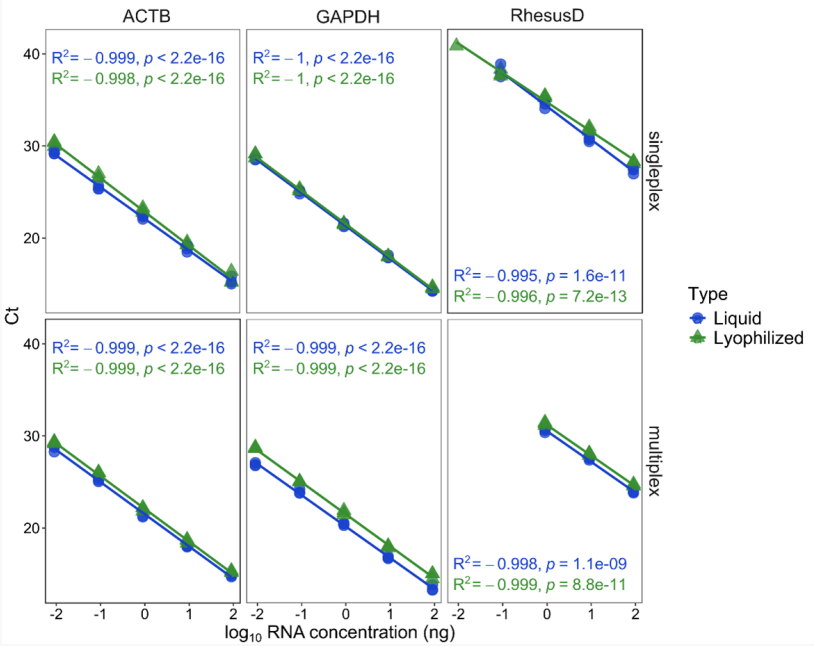

Figure 3: Comparison of standard curves from three genes between singleplex and multiplex qPCR assay using liquid and lyophilized One-Step RT-qPCR master mix. R^2 shows the linearity of Ct values against $\log_{10}$ RNA concentrations and *p* value represents significance of it.

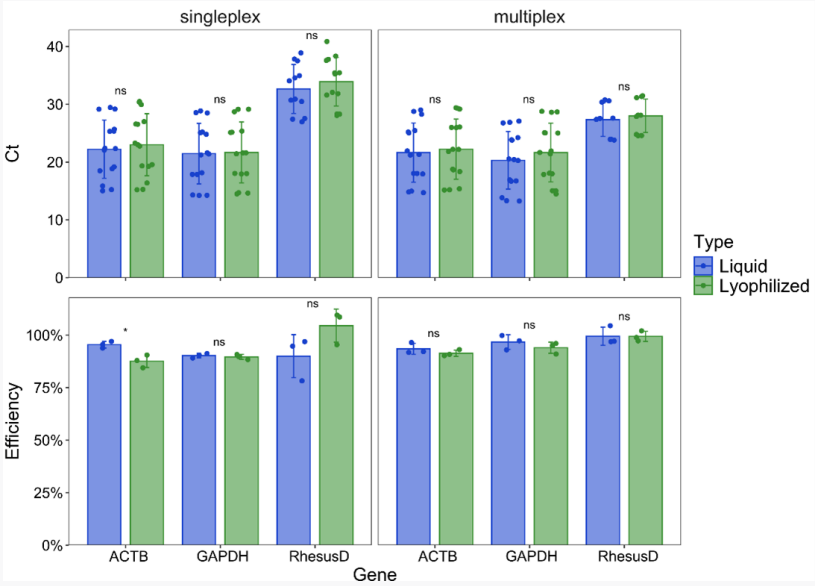

Figure 4: Comparison of Ct values and PCR efficiency (%) between singleplex and multiplex qPCR assays using liquid and lyophilized One-Step RT-qPCR master mix. For Ct values, each bar represents the mean values of ≥ three replicates for five different RNA dilutions (single dots). PCR efficiency is displayed as mean values (bars) and per replicate (dots). Mean Ct and efficiency values of liquid and lyophilized groups of genes within singleplex and multiplex assays were statistically tested using a two-way ANOVA (ns = not significant, * = $p < 0.05$).

Summary

Lyophilized qPCR master mix represents an adequate alternative to conventional liquid reagent mix since it requires less access to cooling facilities, which makes it more robust for transport and increases the shelf-life for many applications. In addition, employing One-Step RT-qPCR mix, RNA analysis can be conducted within one workflow process.

To achieve the same consistency and performance for amplification data, a highly reliable qPCR instrument is required. The qTOWERiris fulfils the needed criteria for reproducible, specific, and efficient amplification for both liquid and lyophilized qPCR master mix regardless of the plate format and multiplex capabilities.

Figure 5: qTOWERiris

Recommended device configuration

Table 4: Overview of devices, accessories, and consumables

Article	Article number	Description
qTOWERiris incl. Color Module 1	844-00853-x*	Real-time PCR system designed in the standard 96-well format, operable via PC, customizable with up to 6 color modules. Available as 100 V, 115 V, and 230 V version.
qTOWERiris 384 incl. Color Module 1	844-00858-x*	Real-time PCR system designed for high-throughput 384-well format, operable via PC, customizable with up to 6 color modules. Available as 100 V, 115 V, and 230 V version.
Color Module 2	844-00821-0	Color module for the excitation and emission of fluorescence dyes like JOE™, HEX™, VIC®, YakimaYellow® or TET™.
Color Module 4	844-00823-0	Color module for the excitation and emission of fluorescence dyes like ROX™, TexasRed®, Cy3.5® or ATTO590.
Color Module 5	844-00824-0	Color module for the excitation and emission of fluorescence dyes like Cy5® or ATTO633.

*x=2 for 230 V, x=4 for 115 V and x=5 for 100 V

References

[1] Choice Of One Step RT-qPCR Or Two Step RT-qPCR | NEB. https://www.neb.com/en/applications/dna-amplification-pcr-and-qpcr/choice-of-one-step-rt-qpcr-or-two-step-rt-qpcr

[2] Lee, M.A. Key considerations for optimal lyophilized reagent development.

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