Molecular assay development

Preparation of an air-dried RT-qPCR assay using Dry-Ready reagents

Introduction

The development of stable, dried RT-qPCR assays is crucial for enhancing the shelf-life and robustness of diagnostic tools, particularly in resource-limited settings where cold chain storage is not always feasible. Traditional RT-qPCR assays require careful handling and storage conditions to maintain the activity of key components. Lyophilization, or freeze-drying, is a common method used to stabilize biological assays. However, it involves complex and costly equipment, as well as energy-intensive processes. In contrast, hot air-drying offers a simpler and more cost-effective alternative, but it presents challenges in maintaining the stability and activity of sensitive assay components. Recent advancements in the stabilization of these assays through the use of various excipients and other improvements have shown promise in extending their usability and performance.

Stable, dried RT-qPCR assays have a wide range of applications, including field diagnostics, point-of-care testing, and long-term storage for emergency preparedness. These assays can be used in remote areas where access to sophisticated laboratory equipment and cold storage is limited, enabling rapid and accurate disease detection and monitoring. In addition, they offer significant advantages for shipping and storage, reducing the need for controlled environments and lowering overall costs. By enabling the stability and reliability of RT-qPCR assays under ambient conditions, assay developers and researchers can deploy these tools more effectively in diverse and challenging environments. This application note details our experimental approach, findings, and the potential implications for the widespread use of stable, air-dried RT-qPCR assays in various applications.

Materials and methods

Air-drying using components of the Dry-Ready kit

Components of the Invitrogen™ Dry-Ready™ RT-qPCR Kit were used to prepare 5 µL of an RT-qPCR assay, which was aliquoted into an Applied Biosystems™ MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL (Cat. No. 4346907). The Invitrogen™ Dry-Ready™ reagents used for this assay are listed in Table 1. The assay was then air-dried for 2.5 hours at 50°C using a Memmert IF30 oven. Before RT-qPCR assay testing, the dried samples were reconstituted with 18 µL of nuclease-free water.

Table 1. RT-qPCR assay reagents for air-drying.

Reagent	Volume (µL) for 5 µL assay, dried	Final concentration in reconstituted RT-qPCR assay (20 µL)	Supplier	Cat. No.
Invitrogen™ Dry-Ready™ SuperScript™ III Flash Reverse Transcriptase	0.1	0.3 U/µL	Thermo Fisher Scientific	A40006906
Invitrogen™ Dry-Ready™ Platinum II *Taq* DNA Polymerase	0.12	0.12 U/µL	Thermo Fisher Scientific	
Invitrogen™ Dry-Ready™ RNaseOUT Recombinant Ribonuclease Inhibitor, 40 U/µL	0.2	0.4 U/µL	Thermo Fisher Scientific	
Invitrogen™ Dry-Ready™ dNTP Mix, 100 mM each	See Table 2 for optimal concentrations		Thermo Fisher Scientific	
1 M $MgCl_2$			Thermo Fisher Scientific	
10X Invitrogen™ Dry-Ready™ Buffer for RT-qPCR	2	1X	Thermo Fisher Scientific	
20X primer mix	1	1X	–	–
Thermo Scientific™ Water, nuclease-free	Up to 5	–	Thermo Fisher Scientific	R0581

Air-drying supplemented with an excipient mix

An RT-qPCR assay at 6 µL reaction volume was prepared using Dry-Ready reagents (Table 3), aliquoted into a MicroAmp Fast Optical 96-Well Reaction Plate, 0.1 mL (Cat. No. 4346907), and air-dried for 2.5 hours at 50°C using a Memmert IF30 oven. Before RT-qPCR assay testing, the dried samples were reconstituted with 18 µL of nuclease-free water.

Table 2. Applied Biosystems™ TaqMan™ Assays and optimal $MgCl_2$:dNTP ratios at 4X assay concentration used during air-drying.

Assay	Mg^{2+} ion concentration	Total dNTPs concentration	dNTP:Mg^{2+} ratio	Cat. No.
GAPDH TaqMan Gene Expression Assay	32 mM	20 mM	1:1.6	4485713
RNase P TaqMan Gene Expression Assay	32 mM	20 mM	1:1.6	4485715
MOB1B TaqMan Gene Expression Assay	32 mM	20 mM	1:1.6	4351370
Xeno™ RNA Control TaqMan Gene Expression Assay	20 mM	12.8 mM	1:1.6	AC00010014

Table 3. RT-qPCR assay reagents for air-drying with excipient mix.

Reagent	Volume (µL) for 6 µL assay, dried	Final concentration in reconstituted RT-qPCR assay (20 µL)	Supplier	Cat. No.
Dry-Ready SuperScript III Flash Reverse Transcriptase	0.1	1 U/µL	Thermo Fisher Scientific	Available as custom products. See thermofisher.com/mdx for ordering information.
Dry-Ready Platinum II *Taq* DNA Polymerase	0.12	0.1 U/µL	Thermo Fisher Scientific	
Dry-Ready RNaseOUT Recombinant Ribonuclease Inhibitor, 40 U/µL	0.2	0.4 U/µL	Thermo Fisher Scientific	
Dry-Ready dNTP Mix, 100 mM each	See Table 2 for optimal concentrations		Thermo Fisher Scientific	
1 M $MgCl_2$			Thermo Fisher Scientific	
10X Dry-Ready Excipient Mix	2	1X	Thermo Fisher Scientific	
10X reaction buffer	2	1X	Thermo Fisher Scientific	
20X primer mix	1	1X	–	–
Water, nuclease-free	Up to 6	–	Thermo Fisher Scientific	R0581

Table 4. Lyophilization reagents.

Reagent	Final concentration for RT-qPCR assay	Supplier	Cat. No.
Invitrogen™ Lyo-Ready™ SuperScript™ III Flash Reverse Transcriptase	1.2 U/µL	Thermo Fisher Scientific	A40006521
Invitrogen™ Lyo-Ready™ Platinum II *Taq* DNA Polymerase	0.4 U/µL	Thermo Fisher Scientific	Available as custom products. See thermofisher.com/mdx for ordering information.
Invitrogen™ Lyo-Ready™ RNaseOUT RNase Inhibitor, 100 U/µL	2 U/µL	Thermo Fisher Scientific	
dNTP mix, 10 mM each	0.15 mM (each)	Thermo Fisher Scientific	R0191
$MgCl_2$	10 mM	Thermo Fisher Scientific	R0971
Reaction buffer for RT-qPCR with excipients	1X	Thermo Fisher Scientific	NA
20X primer/probe mix	1X	–	See Table 2

Lyophilization

An RT-qPCR assay at 20 µL reaction volume was prepared using Invitrogen™ Lyo-Ready™ reagents (Table 4). It was then aliquoted into a Thermo Scientific™ Armadillo™ Low-Profile PCR Strip Plate, 96 well (Cat. No. AB2696), and lyophilized using a Telstar™ LyoBeta™ 25 lyophilizer according to the parameters in Table 5.

As dried samples are extremely sensitive to ambient moisture, the lyophilized and air-dried samples were carefully sealed in moisture- and air-tight bags (Landsberg Buena Park #SS700) using a Gandus Saldatrici bag sealer with fresh silica gel desiccant (>1 g/plate) to remove any leftover moisture, and stored at 50°C for an accelerated stability test. Before one-step RT-qPCR testing, the samples were reconstituted with 18 µL of nuclease-free water.

Table 5. Lyophilization parameters.

Step	Temperature (°C)	Pressure (mBar)	Time (h:min)
Pre-freezing	–60	NA	2:00
Primary drying	–60	0.133	0:15
	–35	0.067	0:00
	–35	0.067	10:00
Secondary drying	–10	0.067	5:00
	–10	0.067	0:10
	20	0.067	3:30
	20	0.067	4:00
	25	0.133	0:00
	25	0.133	2:00

RT-qPCR assay

The one-step RT-qPCR reaction mix for the detection of various targets (Table 2) using Invitrogen™ Universal Human Reference RNA (UHRR) (Cat. No. QS0639) or Applied Biosystems™ TaqMan™ Universal RNA Spike In/Reverse Transcription (Xeno) Control was prepared on ice. Prepared RNA at a volume of 2 µL was added to 18 µL of reconstituted air-dried assay, resulting in 20 µL of final RT-qPCR volume. Sterile-filtered pipette tips were used to prevent aerosol contamination.

RT-qPCR reactions were performed using an Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System. RT-qPCR was performed as a one-step protocol, with both reverse transcriptase and DNA polymerase in the same tube, as shown in Table 6.

Table 6. One-step cycling protocol for RT-qPCR.

Number of cycles	Step	Temperature	Time per cycle
1	Reverse transcription: Dry-Ready SuperScript III **Flash** Reverse Transcriptase	60°C	1 min
	OR Reverse transcription: Dry-Ready SuperScript III Reverse Transcriptase	50°C	5 min
	Initial denaturation	95°C	2 min
40	Denaturation	95°C	1 s
40	Amplification	60°C	20 s

Results

Exceptional stability of air-dried assay: Xeno RNA assay

Our experiments aimed to evaluate the stability and performance of hot air-dried RT-qPCR assays. The assays were subjected to hot air drying, followed by storage at increased temperatures for extended periods to assess the long-term stability of the assays. To demonstrate the stability of the assay prepared using Dry-Ready kit components, we used the Dry-Ready RT-qPCR Kit (Cat. No. A40006906) to prepare Xeno RNA Control TaqMan Gene Expression Assay for the detection of the TaqMan Universal RNA Spike In/Reverse Transcription (Xeno) Control. This assay was air-dried and subjected to a stability study at an elevated temperature of 50°C (Figure 1) for 10 weeks. Our findings showed that the optimized Dry-Ready assay composition exhibited excellent stability under these conditions, with no worsening of C_t values compared to a freshly prepared non-dried assay. Extrapolation of these results (according to the Van't Hoff rule) suggested that the air-dried assay can remain stable for at least one year at ambient temperatures.

Another important aspect for the maximum stability of the dried assays was optimizing the ratio of dNTP:$MgCl_2$. Results showed (Figure 2) that initially, after air-drying, both tested assays (with 20 mM $MgCl_2$ and 40 mM $MgCl_2$) performed similarly. However, after storing the assays for 4 weeks at 50°C (which can be extrapolated into approximately 10 weeks at ambient temperature), the 40 mM magnesium ion concentration (representing the 1:3.1 dNTP:Mg^{2+} ratio) was no longer suitable for efficient RT-qPCR analysis. Only the assay with a dNTP:Mg^{2+} ratio of 1:1.6 remained stable for the duration of the experiment. These data showed that a balanced ratio prevented assay degradation and enabled efficient amplification, resulting in consistent C_t values and robust amplification curves. Therefore, prior to further assay development, different dNTP:$MgCl_2$ ratios should be tested by analyzing air-dried assays after 1 week of incubation at 50°C before proceeding to long-term stability tests (see Table 2 for optimal dNTP:$MgCl_2$ ratios used for further testing).

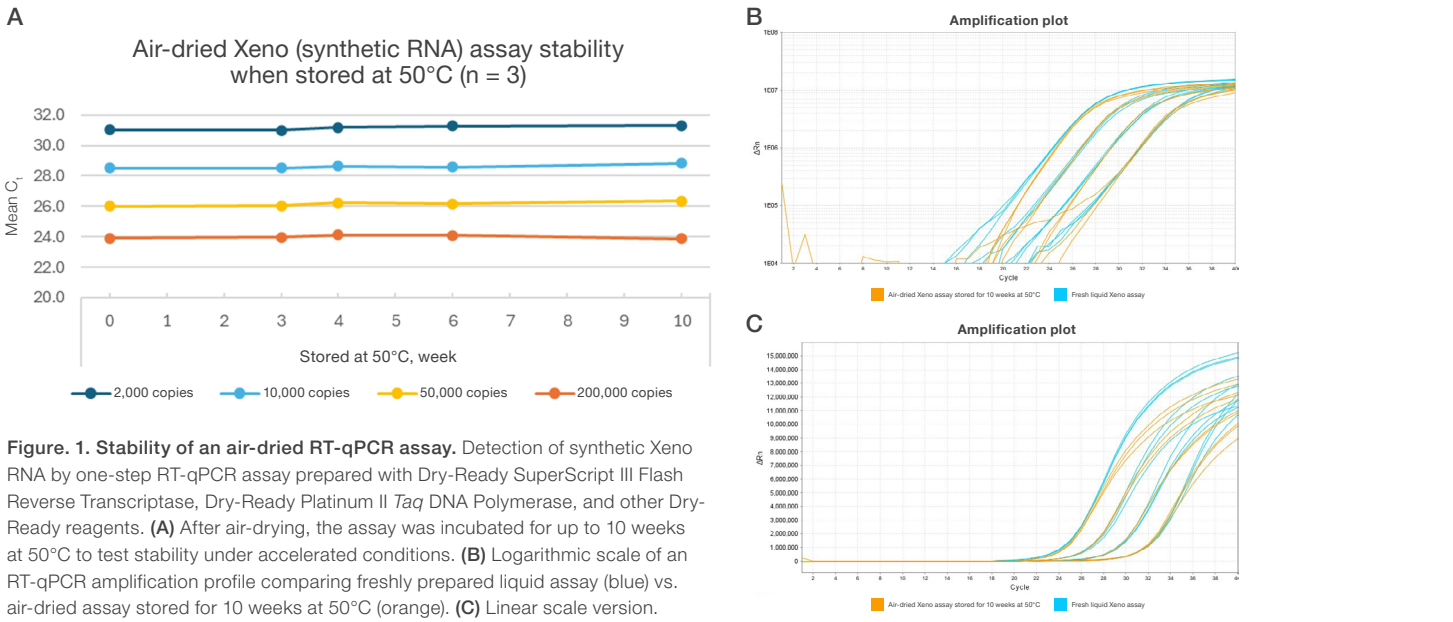


Figure. 1. Stability of an air-dried RT-qPCR assay. Detection of synthetic Xeno RNA by one-step RT-qPCR assay prepared with Dry-Ready SuperScript III Flash Reverse Transcriptase, Dry-Ready Platinum II *Taq* DNA Polymerase, and other Dry-Ready reagents. **(A)** After air-drying, the assay was incubated for up to 10 weeks at 50°C to test stability under accelerated conditions. **(B)** Logarithmic scale of an RT-qPCR amplification profile comparing freshly prepared liquid assay (blue) vs. air-dried assay stored for 10 weeks at 50°C (orange). **(C)** Linear scale version.

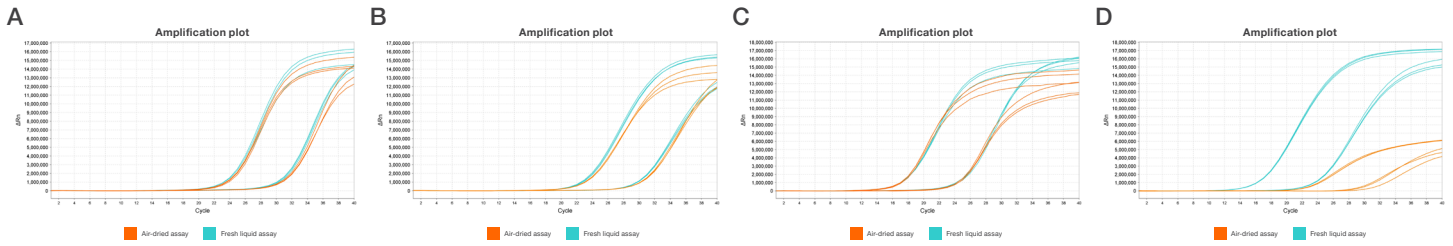
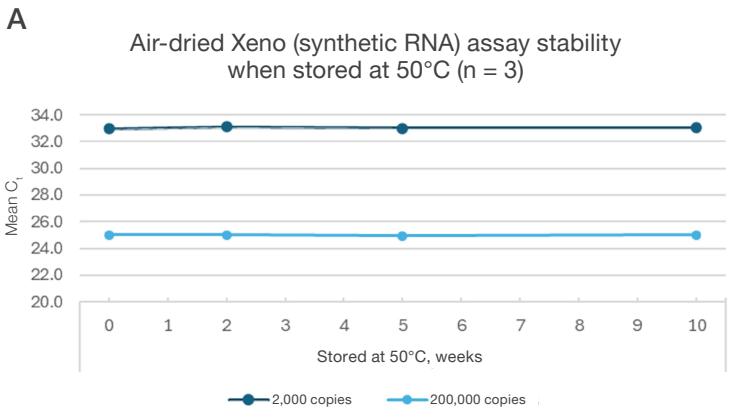


Figure 2. Xeno RNA detection assay stability using different dNTP:$MgCl_2$ ratios. (A) Assay using 20 mM $MgCl_2$ initially after air drying (orange) vs. freshly prepared liquid assay (blue). **(B)** Assay using 40 mM $MgCl_2$ initially after air drying (orange) vs. freshly prepared liquid assay (blue). **(C)** Assay using 20 mM $MgCl_2$ after air drying and storing for 4 weeks at 50°C (orange) vs. freshly prepared liquid assay (blue). **(D)** Assay using 40 mM $MgCl_2$ after air drying and storing for 4 weeks at 50°C (orange) vs. freshly prepared liquid assay (blue).

To further explore the ability of Dry-Ready kit components to be used in different assay settings, we prepared a Xeno RNA Control TaqMan Gene Expression Assay using Dry-Ready SuperScript III Reverse Transcriptase combined with Dry-Ready Platinum II *Taq* DNA Polymerase and supplemented with 10X Dry-Ready Excipient Mix. The use of specific stabilizers included in this excipient mix significantly improved the stability of the dried RT-qPCR assays (Figure 3). The inclusion of these stabilizers resulted in enhanced preservation of assay activity and integrity, leading to prolonged assay stability up to one year at room temperature.

Stability of air-dried assays using human RNA detection systems

While the Xeno RNA Control TaqMan Gene Expression Assay for synthetic Xeno RNA was very sensitive to changes in one-step RT-qPCR assay performance during air-drying, the next step in our work was to analyze the use of the air-drying protocol with three different primer systems targeting human RNA transcripts. As with the previous experiment, we air-dried GAPDH, RNase P, and MOB1B detection assays and left them for 20 days at 50°C for stability and performance tests (Figure 4). The resulting data confirmed our previous findings that all these assays retained excellent stability with no change in C_t values during storage.



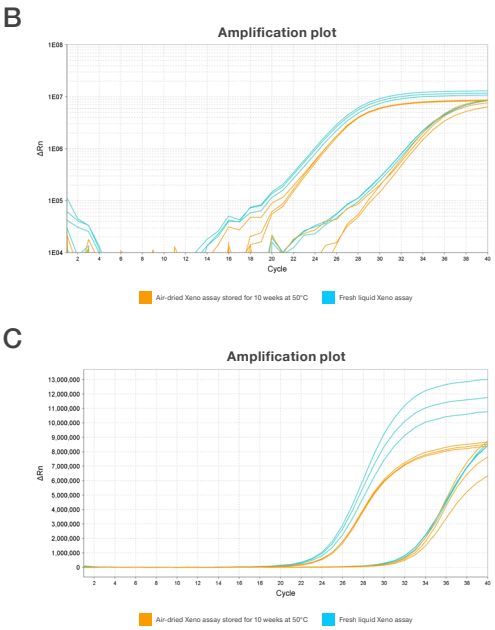


Figure 3. Stability of air-dried RT-qPCR assay. Detection of synthetic Xeno RNA substrate by one-step RT-qPCR assay prepared with Dry-Ready SuperScript III Reverse Transcriptase, Dry-Ready Platinum II *Taq* DNA Polymerase, other Dry-Ready reagents, and supplemented with Dry-Ready Excipient Mix. **(A)** After air-drying, the assay was incubated up to 10 weeks at 50°C to test stability at accelerated conditions. **(B)** Logarithmic scale of RT-qPCR amplification profile comparing freshly prepared liquid assay (blue) vs. air-dried assay stored for 10 weeks at 50°C (orange). **(C)** Linear scale version.

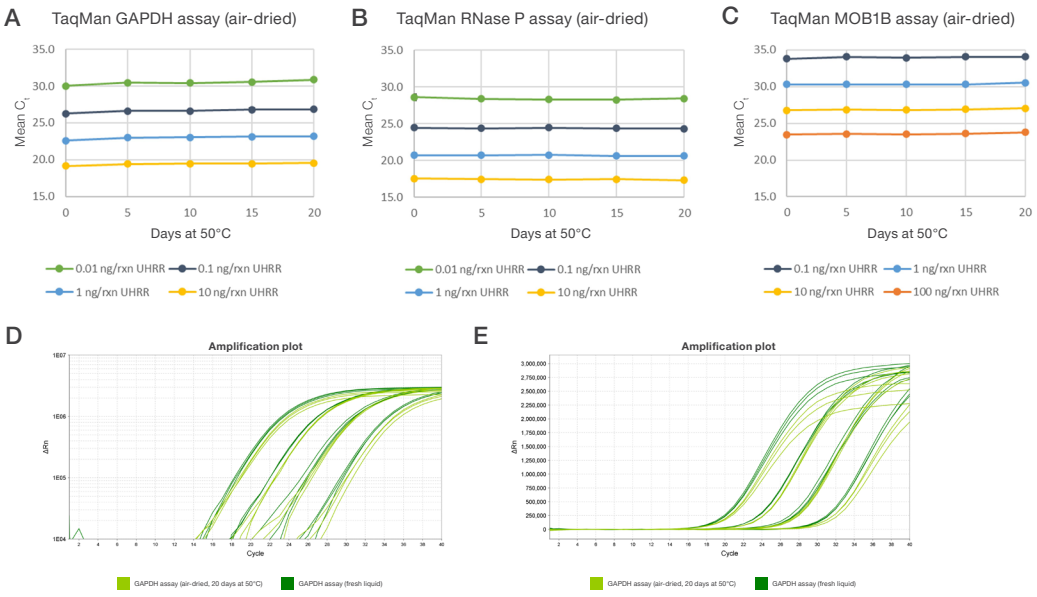


Figure 4. Stability of air-dried RT-qPCR assay. Detection of GAPDH, RNase P, and MOB1B transcripts of the human transcriptome (series of ten-fold dilutions) by one-step RT-qPCR assay prepared with Dry-Ready SuperScript III Flash Reverse Transcriptase, Dry-Ready Platinum II *Taq* DNA Polymerase, and other Dry-Ready reagents. After air-drying, the assays were incubated up to 20 days at 50°C to test stability at accelerated conditions. **(A)** Air-dried TaqMan GAPDH assay. **(B)** Air-dried TaqMan RNase P assay. **(C)** Air-dried TaqMan MOB1B assay. **(D)** Logarithmic scale of RT-qPCR amplification profile of the GAPDH assay comparing freshly prepared liquid assay (dark green) vs. air-dried assay stored for 20 days at 50°C (light green). **(E)** Linear scale version.

Air-drying vs. lyophilization: 3-plex assay

To gain more insights into the newly developed air-drying protocol, we also compared assay performance against lyophilized assays. This time, GAPDH, RNase P, and MOB1B detection assays were combined into a triplex assay, as such systems are usually more challenging than singleplex systems. Comparing the performance of qPCR assays subjected to hot air-drying versus lyophilization revealed that, while both air-dried and lyophilized assays offered excellent stability (Figure 5), lyophilization involved higher costs, a longer protocol, and a custom assay formulation. Hot air-drying, when optimized, provided a cost-effective and efficient alternative, maintaining assay performance over extended storage periods. The optimized hot air–dried assays demonstrated C_t values and amplification curves comparable to those of lyophilized assays.

These results demonstrate that careful selection of stabilizers, buffer systems, and the optimization of component ratios are crucial for developing stable dried qPCR assays. The ability to maintain assay performance after hot air-drying and prolonged storage under ambient conditions underscores the potential of these assays for use in diverse applications.

Conclusion

The findings from our study highlight the potential of stable dried qPCR assays for use in diverse applications, particularly in settings where cold chain storage is not feasible. By developing optimized Dry-Ready reagents, we have demonstrated that it is possible to achieve robust and reliable one-step RT-qPCR assays that remain stable under ambient conditions for at least one year.

These stable, dried RT-qPCR assays can be deployed in field diagnostics, point-of-care testing, and emergency preparedness, offering rapid and accurate disease detection in remote and resource-limited areas. The ability to maintain assay performance without the need for controlled storage environments significantly reduces costs and logistical challenges, making these assays highly practical for widespread use.

Our results underscore the importance of careful formulation and optimization in the development of dried RT-qPCR assays. By leveraging advanced compositions, we can help ensure the long-term stability and reliability of these diagnostic tools, ultimately enhancing their utility and impact in various healthcare and research settings.

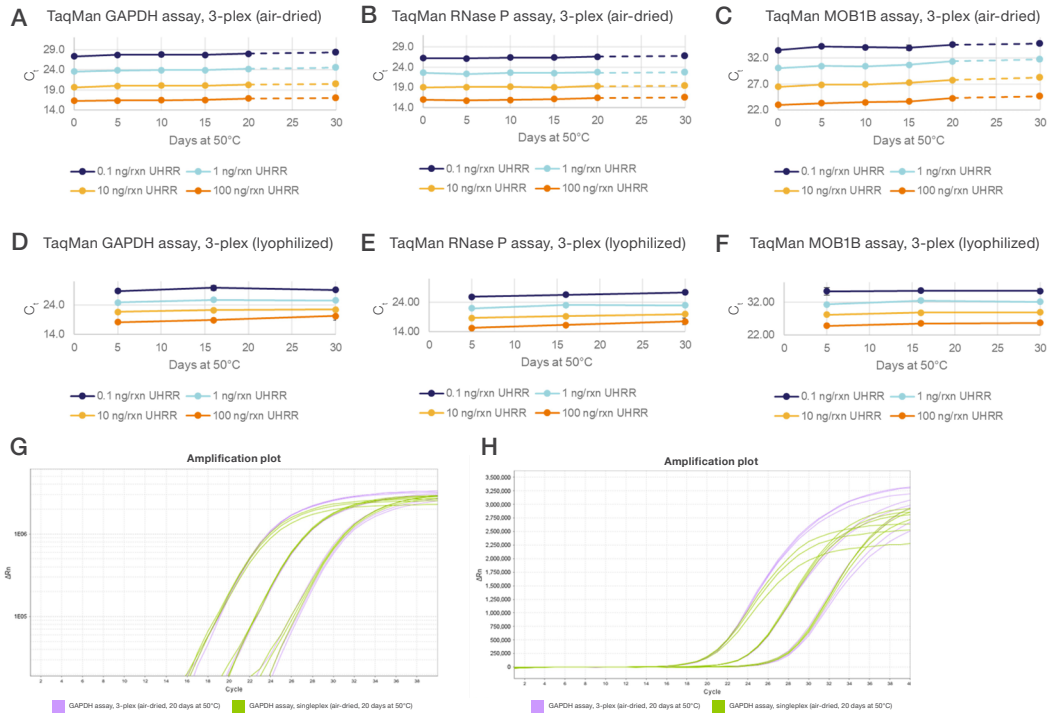


Figure 5. Stability of air-dried triplex RT-qPCR assay compared with lyophilized assay. Detection of GAPDH, RNase P, and MOB1B transcripts of the human transcriptome (series of ten-fold dilutions) by multiplex one-step RT-qPCR assay prepared with Dry-Ready SuperScript III Flash Reverse Transcriptase, Dry-Ready Platinum II *Taq* DNA Polymerase, and other Dry-Ready reagents. After air-drying, the assay was incubated up to 20 days at 50°C to test stability under accelerated conditions. **(A)** Air-dried TaqMan GAPDH assay. **(B)** Air-dried TaqMan RNase P assay. **(C)** Air-dried TaqMan MOB1B assay. **(D)** Detection of GAPDH transcripts of the human transcriptome by multiplex one-step RT-qPCR assay prepared with Lyo-Ready SuperScript III Reverse transcriptase, Lyo-Ready Platinum II *Taq* DNA polymerase, and other Lyo-Ready reagents. **(E)** Detection of RNase P. **(F)** Detection of MOB1B. **(G)** Logarithmic scale of RT-qPCR amplification profile of air-dried GAPDH assay from multiplex detection (purple) vs. assay using singleplex detection (light green). **(H)** Linear scale version.

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