Example Report: Preservation of Influenza RT-PCR Assays

1 - Summary

PCRstable® technology is used for developing chemical stabilizer alternatives to lyophilization, reducing assay development times and improving assay workflows. Drying with PCRstable® formulations produces ambient-stable reagents ready for testing, shipment and storage worldwide. In this study, we use the PCRstable® technology to stabilize a real-time influenza molecular assay with proprietary compounds in a simple, air dried format. Following accelerated aging procedures at elevated temperatures, we perform real-time RT-PCR assays for detection of influenza RNA to assess the performance of the assays. We demonstrate that the PCRstable® technology successfully preserves the complete assay for at least one year at ambient temperatures, based on accelerated aging calculations.

2 - Background

Biomatrica was founded on the idea that chemistry could be used to replicate the biological process of anhydrobiosis, which protects certain animals hibernating for extended periods and under extreme conditions. The goal of our technology is to be able to protect and preserve biological materials, thereby allowing their use in settings not previously possible. Notable examples of technology include the preservation of assays in microfluidic devices and the protection of unstable targets in liquid biopsy samples.

The typical workflow for developing new preservation formulations is outlined below.

The primary and secondary screening of preservation formulations uses a process similar to combinatorial chemistry in identifying new drugs. Biomatrica has established a library of over a thousand different chemicals that are applied in this screen, enabling us to identify solutions to most customer reagent preservation, positioning and release problems. As each assay is unique, formulations often must be specifically developed and optimized for each customer's assay or reagent.

The duration of the process depends on the type of material being purified, Biomatrica's experience with similar materials, the requirements for a project, and other factors that are determined before a project is initiated. Once a formulation has been established, it will often be transferred to a partner for further product development and ultimately product commercialization.

3 - Material and Methods

Reagent Preparation

Two influenza quadplex real time RT-PCR assays were prepared. One assay mix was prepared with the following components: 2U SuperScript® III reverse transcriptase (Thermo Fisher Scientific), 0.5U GoTaq® DNA polymerase (Promega), RT-PCR buffer, 150µM dATP, dCTP, dGTP, and dTTP, 0.4µM of each primer, and 0.1µM of each fluorescent probe. The second assay master mix was prepared as above except with 2U GoScript® reverse transcriptase (Promega) used in place of SuperScript® III. Mixes were set up containing the enzyme plus one or more of the above listed components, as noted in the figure legends.

Four primer/probe sets were used that target influenza A matrix (InfA universal), influenza B nucleoprotein (InfB universal), and influenza A subtypes H1 (InfH1) and H3 (InfH3).

PCRstable® Stabilizers A, B, and C were added to separate PCR master mixes at a 1:1 (vol:vol) ratio. Each reaction was set up in triplicate, with a final reaction volume of 10 µL.

RNA was purified using the QIAamp® MinElute Virus Spin Kit (Qiagen) from BPL-inactivated influenza. Influenza A H3N2 (A/Perth/16/2009), H1N1 (A/California/7/2009) and Influenza B (B/Brisbane/60/2008) were obtained from Virapur (San Diego, CA). Influenza A H3N2 (A/Fujian/411/2002), H1N1 (A/California/7/2009 NYMC X-179A) and Influenza B (B/Brisbane/60/2008) wer obtained from the Influenza Reagent Resource (CDC).

Drying and Storage

Assay reagents mixed with PCRstable® stabilizers and Non Protected controls (NP) were dried for 1 hour in 96-well PCR plates using a Vacufuge® vacuum concentrator (Eppendorf). Dried reactions were stored with desiccants in moisture barrier bags at 45°C.

Rehydration and Analysis

At 60 days (2 months) of storage, the assay reagents were rehydrated with 10 µL of influenza RNA template at 10x LOD, along with any other assay components not included when the assay was stabilized. Fresh positive control reactions were set up using the same reagent and template concentrations as the dried reactions. All samples were amplified on a LightCycler® 96 Real-Time PCR Instrument (Roche) using the following cycling conditions: 42°C for 30 minutes, 95°C for 5 minutes, and 45 cycles of 95°C for 20 seconds and 60°C for 40 seconds. Endpoint fluorescence was measured using the LightCycler® 96 following real-time PCR.

4 – Results

For initial formulation development, preservation mixtures were screened to identify formulas that would protect a dried mixture of Superscript III reverse transcriptase, GoTaq® DNA polymerase, and dNTPs. Remaining influenza assays components (buffers, primers, and probes), as well as water and RNA template, were used to rehydrate assays following storage (Figure 1), with assay performances tested in qRT-PCR reaction. Once formulas were identified that stabilized these mixtures, they were further screened for their ability to preserve more complex dried assay mixtures that included Superscript® III reverse transcriptase, GoTaq® DNA polymerase, dNTPs, primers, and probes (Figure 2), with water, RNA template, and buffer used to rehydrate those reactions prior to testing.

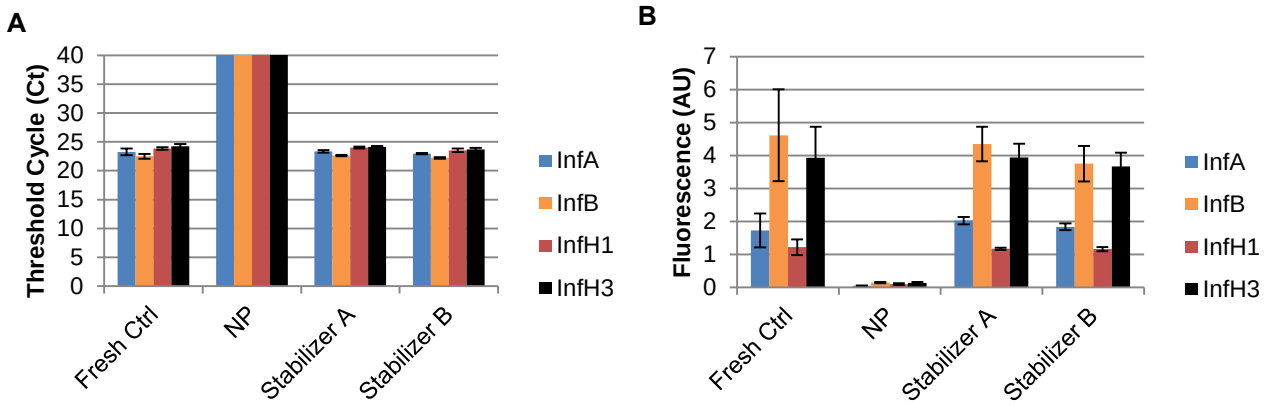

Figure 1: Influenza real-time RT-PCR reactions from dry stabilized SuperScript® III reverse transcriptase, GoTaq® DNA polymerase and dNTPs. *SuperScript® III, GoTaq® and dNTPs were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template, buffer, primers, probes, and water. Fresh represents the positive control, NP represents Non Protected control, and Stabilizer A and B represent stabilized reactions. Error bars represent the standard deviation from triplicate reactions. (A) Cycle times of PCR reactions. Cts of 45 were assigned to any sample that failed to amplify. (B) Fluorescence of PCR reactions following amplification in arbitrary fluorescence units (AU).*

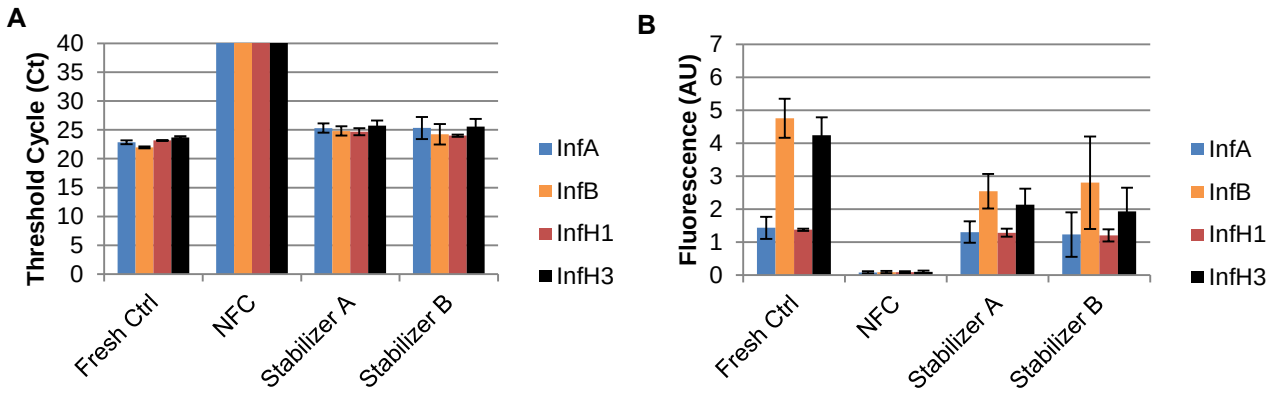

Figure 2: Influenza real-time RT-PCR reactions from dry stabilized SuperScript® III reverse transcriptase, GoTaq® DNA polymerase, dNTPs, plus primers & probes. *SuperScript III, GoTaq, dNTPs, primers, and probes were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template, buffer, and water. Fresh represents the positive control, NP represents Non Protected control, and Stabilizer C and D represent stabilized reactions. Error bars represent the standard deviation from triplicate reactions. (A) Cycle times of PCR reactions. Cts of 45 were assigned to any sample that failed to amplify. (B) Fluorescence of PCR reactions following amplification in arbitrary fluorescence units (AU).*

Following the development of stabilizing formulations for the influenza assays using Superscript® III reverse transcriptase, an additional screen was performed to identify formulations that could preserve assays where GoScript® reverse transcriptase had been used to replace the SuperScript® III enzyme. These formulas were tested using assays that contained the reverse transcriptase, polymerase, and dNTPs (Figure 3), as well as assays with those components plus the assay primers and probes (Figure 4). Assays were rehydrated using buffers, primers, probes, water, and RNA template (Figure 3) or buffer, water, and RNA template (Figure 4) prior to testing in qRT-PCR reactions.

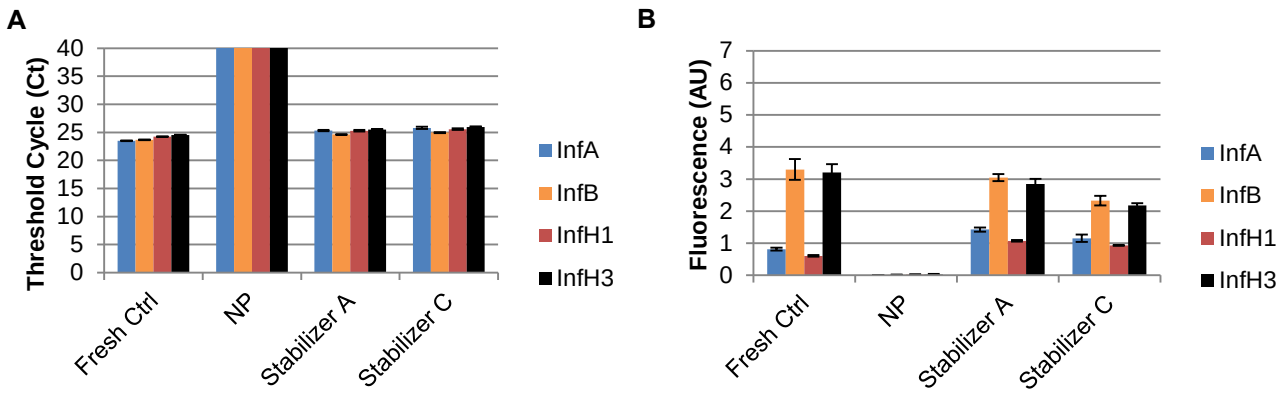

Figure 3: Influenza real-time RT-PCR reactions from dry stabilized GoScript reverse transcriptase, GoTaq® DNA polymerase and dNTPs. *GoScript®, GoTaq® and dNTPs were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template, buffer, primers, probes, and water. Fresh represents the positive control, NP represents Non Protected control, and Stabilizer A and B represent stabilized reactions. Error bars represent the standard deviation from triplicate reactions. (A) Cycle times of PCR reactions. Cts of 45 were assigned to any sample that failed to amplify. (B) Fluorescence of PCR reactions following amplification in arbitrary fluorescence units (AU).*

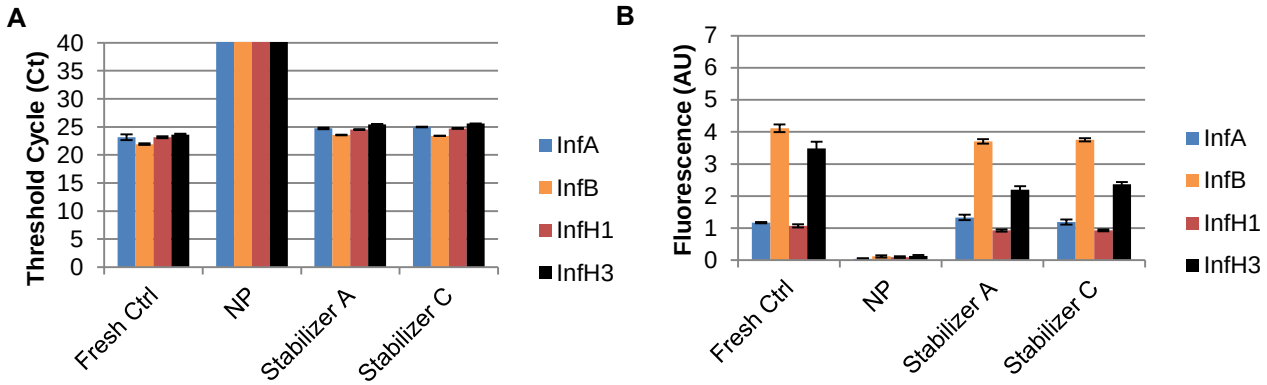

Figure 4: Influenza real-time RT-PCR reactions from dry stabilized GoScript® reverse transcriptase, GoTaq® DNA polymerase, dNTPs, plus primers & probes. *GoScript®, GoTaq®, dNTPs, primers, and probes were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template, buffer, and water. Fresh represents the positive control, NP represents Non Protected control, and Stabilizer C and D represent stabilized reactions. Error bars represent the standard deviation from triplicate reactions. (A) Cycle times of PCR reactions. Cts of 45 were assigned to any sample that failed to amplify. (B) Fluorescence of PCR reactions following amplification in arbitrary fluorescence units (AU).*

5 – Conclusions

The data presented in Figures 1 and 2 shows that real-time RT-PCR reactions using stabilized SuperScript® III reverse transcriptase and GoTaq® DNA Polymerase retain their efficiency to amplify four influenza target RNAs after being dried in the presence of PCRstable® preservation solutions and stored for 2 months at 45°C (equivalent to 12 months at 25°C). Both the cycle times and endpoint fluorescence are comparable to freshly prepared controls. Figures 3 and 4 show that similar performance can be achieved when using GoScript® reverse transcriptase in place of SuperScript® III reverse transcriptase. The data presented demonstrates that RT-PCR assay reagents stabilized by PCRstable® have similar performance to assay reagents prepared from frozen stocks, providing an all-ambient alternative to frozen storage for molecular diagnostic assays. Overall, these results demonstrate the ability of the PCRstable® technology to be used to screen for novel formulations that can protect and preserve complex mixtures.