Influenza real-time RT-PCR assays stabilized with PCRstable® technology

INTRODUCTION

PCRstable is a service for developing chemical stabilizer alternatives to lyophilization, reducing assay development times and improving assay workflows. Drying with the stabilizers 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 biostability 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 stabilized assay. We demonstrate that the PCRstable technology successfully stabilizes the complete assay for at least one year at ambient temperatures, based on accelerated aging calculations.

MATERIALS 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) were 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 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) was 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 LightCycler96 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 LightCycler96 following real-time PCR.

RESULTS

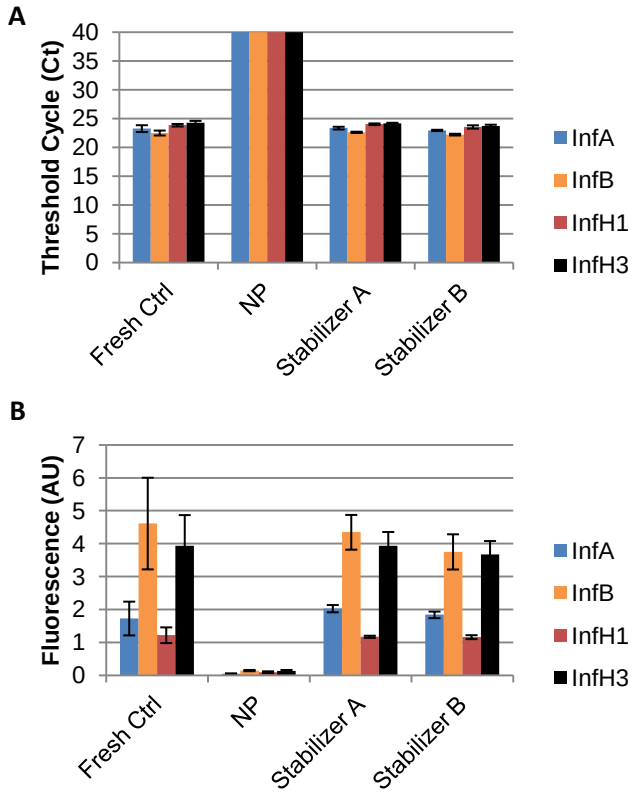
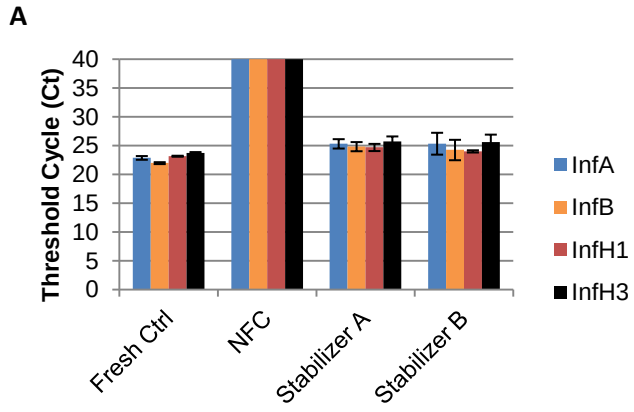


Figure 1: Influenza Real-time RT-PCR reactions from dry stabilized SuperScript III reverse transcriptase, GoTaq DNA polymerase and dNTPs. *SuperScript III, GoTaq and dNTPs were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template, buffer, primers, probes, 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).*



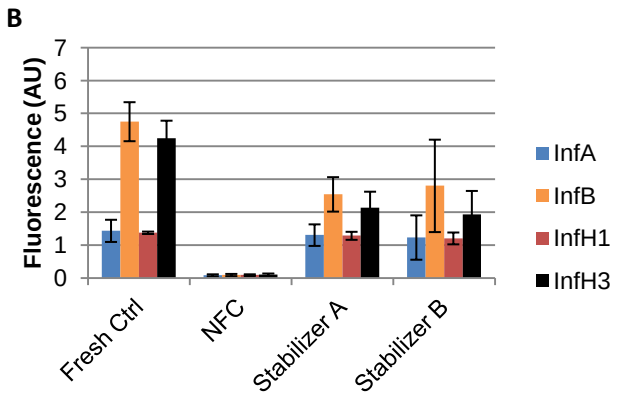


Figure 2: Influenza real-time RT-PCR reactions from dry stabilized SuperScript III reverse transcriptase, GoTaq DNA polymerase, dNTPs, plus primers & probes. *SuperScript III, GoTaq, dNTPs, primers, and probes were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with 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).*

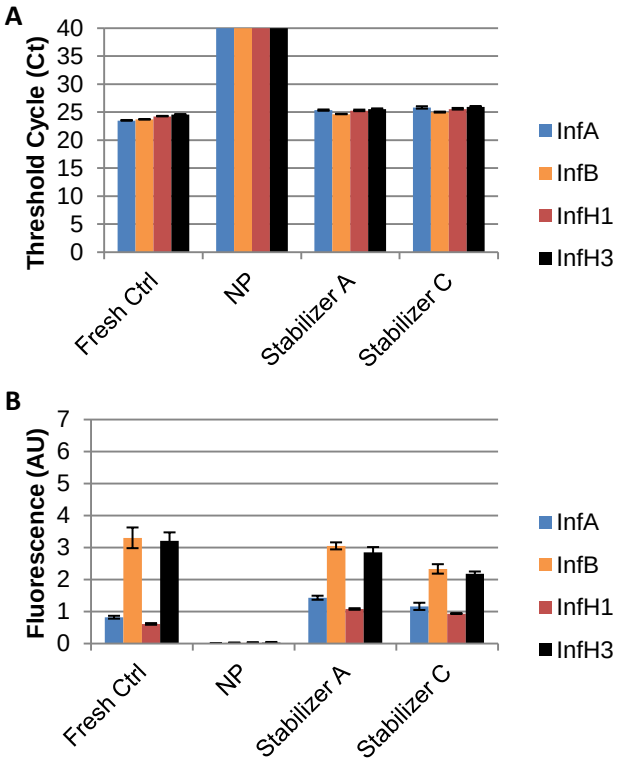


Figure 3: Influenza real-time RT-PCR reactions from dry stabilized GoScript reverse transcriptase, GoTaq DNA polymerase and dNTPs. *GoScript, GoTaq and dNTPs were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template, buffer, primers, probes, and water. 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).*

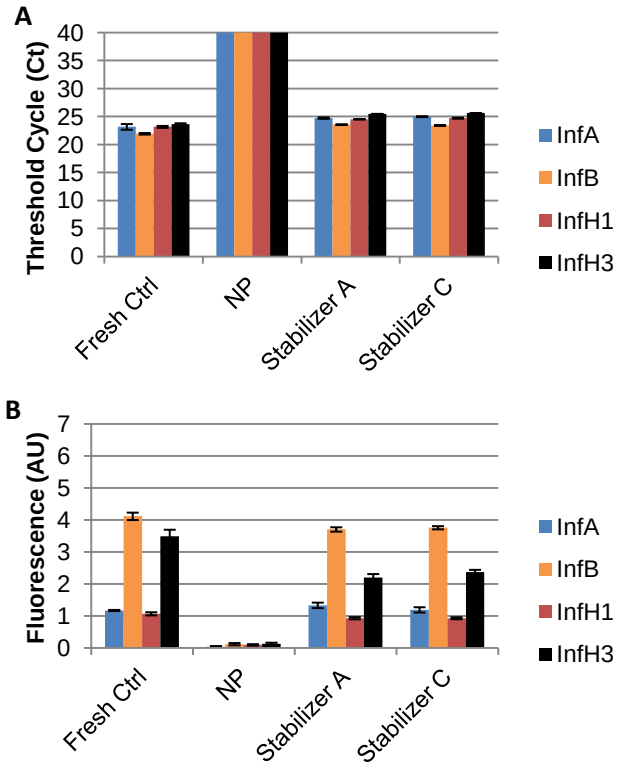


Figure 4: Influenza real-time RT-PCR reactions from dry stabilized GoScript reverse transcriptase, GoTaq DNA polymerase, dNTPs, plus primers & probes. *GoScript, GoTaq, dNTPs, primers, and probes were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template, 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).*

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® stabilizers 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.

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