Ambient stabilization of diagnostic PCR and RT-PCR assays using PCRstable® technology

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Abstract

PCRstable® is a service for developing chemical stabilizer alternatives to lyophilization, reducing assay development time and improving assay workflows. Drying with the stabilizers produces ambient-stable assays ready for testing, shipment and storage worldwide. In this study, we tested the stability and functionality of PCR- and RT-PCR-based, diagnostic, infectious disease assays preserved with PCRstable® solutions using multiple PCR-based reagents from several major vendors.

PCR and RT-PCR assay performance of the stabilized reagents was assessed following accelerated aging studies at elevated temperatures. Using this technology, we show that Superscript III®, GoScript®, GoTaq®, and HawkZ05® enzymes retain full function for at least 1 year at ambient temperatures. Furthermore, we show effective ambient stabilization of these enzymes in a variety of assay formats, including in complete multiplexed diagnostic assays for Influenza, HIV/HCV, and *C. difficile*. Dried reagents were rehydrated with purified RNA/DNA samples and PCR-based assays were conducted.

These methods can be applied to any PCR-based diagnostic method, including infectious disease, cancer, and genetic screening in a variety of formats to simplify assay design, expand distribution, and reduce test complexity.

Materials and Methods

Assay compositions

RT-PCR assays for Influenza and HIV/HCV, and a PCR assay for *C. diff.* were prepared for stabilization testing.

- Influenza assay composition
Two influenza quadplex real time RT-PCR assays were prepared. The first contained 2U SuperScript III reverse transcriptase (Thermo Fisher Scientific) and 0.5U GoTaq DNA polymerase (Promega) per reaction. A separate influenza assay contained 2U GoScript reverse transcriptase (Promega) and 0.5U GoTaq per reaction. Both assays contained RT-PCR buffer, 0.15mM of dATP, dCTP, dGTP, and dTTP, 0.4µM of each primer, and 0.1µM of each fluorescent probe.

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).

- HIV/HCV assay composition 1 (HawkZ05 enzyme separate formats)
Two HIV/HCV duplex real time RT-PCR assays were prepared. One assay mix was prepared with the following components: 2.5U HawkZ05 DNA polymerase (Roche), RT-PCR buffer, 200mM dATP, 200mM dCTP, 200mM dGTP, 300mM dUTP, 30mM dTTP, 1.5mM manganese acetate, 0.4µM of each primer, and 0.1µM of each fluorescent probe. Mixes were set up containing the enzyme plus one or more of the above listed components, as noted in the figure legends.

- HIV/HCV assay composition 2 (HawkZ05 Fast One-step Lyo format)
The second assay master mix was prepared with the following components: HawkZ05 Fast One-step RT-PCR Lyo Kit diluted to 1x (Roche), 0.4µM of each primer, and 0.1µM of each fluorescent probe.

A single primer/probe set for each HIV and HCV target was used.

- *C. diff.* assay composition
Duplex real-time *C. diff* DNA assays were assembled with the following standard qPCR components: buffer, 0.2mM dNTPs, 0.45µM or 0.7µM primers, 0.3µM probes, and 2U GoTaq glycerol free enzyme per reaction.

Two *C diff.* primer/probe sets and a single internal control set were used.

Stabilizer addition, drying and storage
Influenza: 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. 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.

HIV/HCV assays: PCRstable ® Stabilizers A to F 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 10µL. Assay reagents mixed with PCRstable® stabilizers and the 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.

C. diff.: PCRstable® Stabilizers A and B were added to separate PCR master mixes at a 1:1 (vol:vol) ratio. The assays and Non Protected controls (NP) were dried onto 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
Rehydration method (all assays performed at 10x LOD). At 60 days (2 months) of storage, all dry stabilized assay reagent mixtures were rehydrated with 10 µL of 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.

Assay cycling conditions
Influenza: 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.

HIV/HCV: Samples were amplified on a CFX96 Real-Time PCR Instrument (Bio-Rad) using the following cycling conditions: 52°C for 5 minutes, 55°C for 5 minutes, 60°C for 10 minutes, 65°C for 5 minutes, and 45 cycles of 94°C for 10 seconds and 60°C for 1 minute. Endpoint fluorescence was measured using the CFX96 following real-time PCR.

C. diff.: Samples were amplified on a CFX96 Real-Time PCR Instrument (Bio-Rad) using the following cycling conditions: 45 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Endpoint fluorescence was measured using the CFX96 following real-time PCR.

Results

Superscript III + GoTaq (+ dNTPs) stabilization

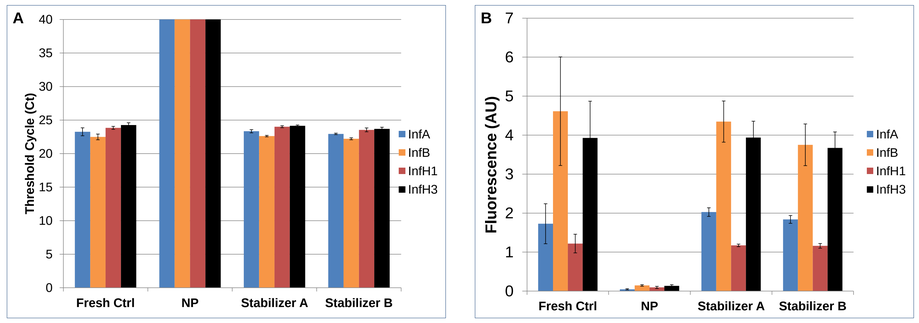

Figure 1: Influenza real-time RT-PCR reactions from dry stabilized SuperScript III reverse transcriptase, GoTaq DNA polymerase and dNTPs. SuperScript III, GoTaq and dNTPs were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template, buffer, primers, probes,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

Superscript III + GoTaq (+ dNTPs, primers and probes) stabilization

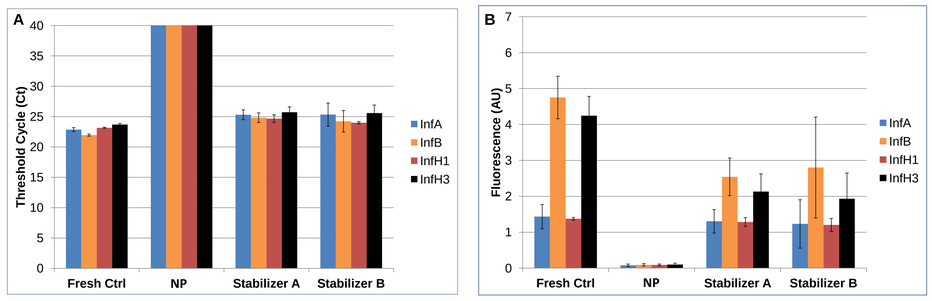

Figure 2: Influenza real-time RT-PCR reactions from dry stabilized SuperScript III reverse transcriptase, GoTaq DNA polymerase, dNTPs, plus primers & probes. SuperScript III, GoTaq, dNTPs, primers, and probes were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with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

GoScript + GoTaq (+ dNTPs) stabilization

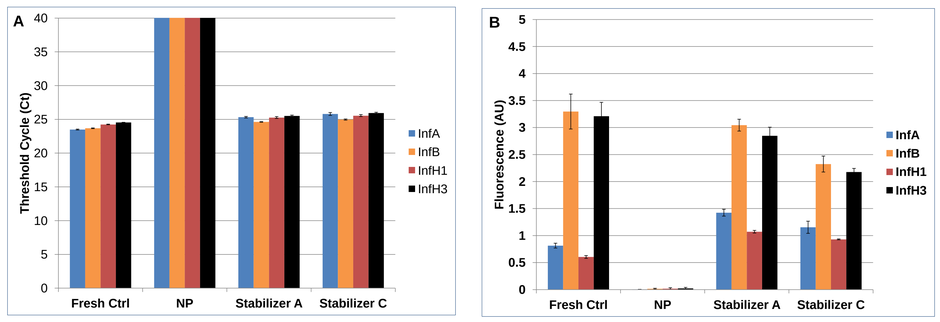

Figure 3: Influenza real-time RT-PCR reactions from dry stabilized GoScript reverse transcriptase, GoTaq DNA polymerase and dNTPs. GoScript, GoTaq and dNTPs were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template, buffer, primers, probes, and water.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

GoScript + GoTaq (+ dNTPs, primers and probes) stabilization

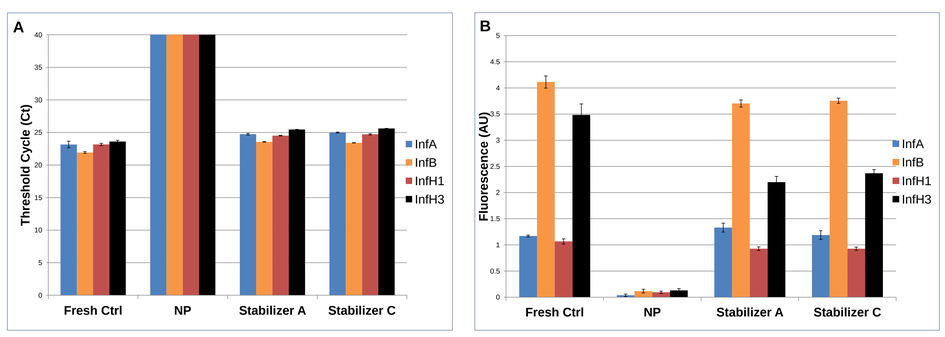

Figure 4: Influenza real-time RT-PCR reactions from dry stabilized GoScript reverse transcriptase, GoTaq DNA polymerase, dNTPs, plus primers & probes. GoScript, GoTaq, dNTPs, primers, and probes were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template,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

HawkZ05 (+ dNTPs) stabilization

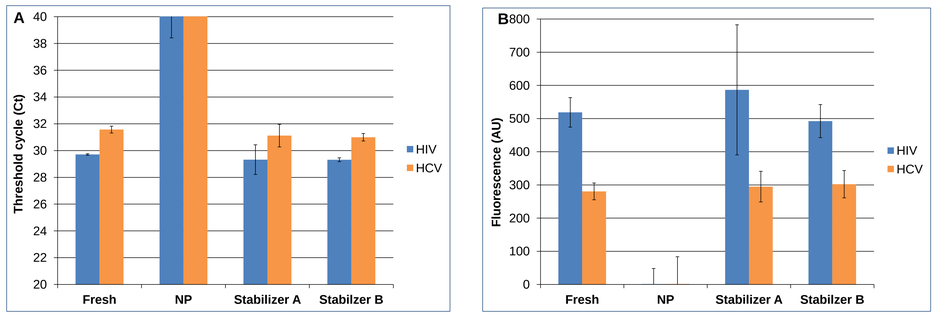

Figure 5: HIV/HCV RT-PCR reactions from dry stabilized HawkZ05 DNA polymerase and dNTPs. HawkZ05 enzyme and dNTPs were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template, buffer, primers, probes, and water. Fresh represents the positive control, NP 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).

HawkZ05 (+ dNTPs, primers and probes) stabilization

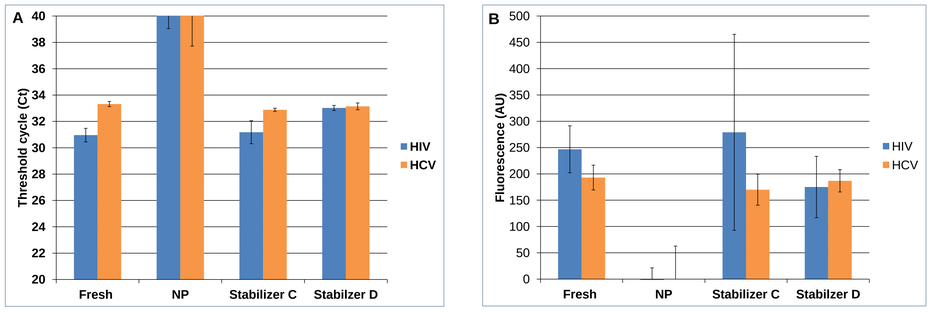

Figure 6: HIV/HCV RT-PCR reactions from dry stabilized HawkZ05 DNA polymerase, dNTPs, and primers & probes. HawkZ05 enzyme, dNTPs, primers, and probes were stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template, buffer, and water. Fresh represents the positive 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).

HawkZ05 Fast One-Step Lyo complete assay stabilization

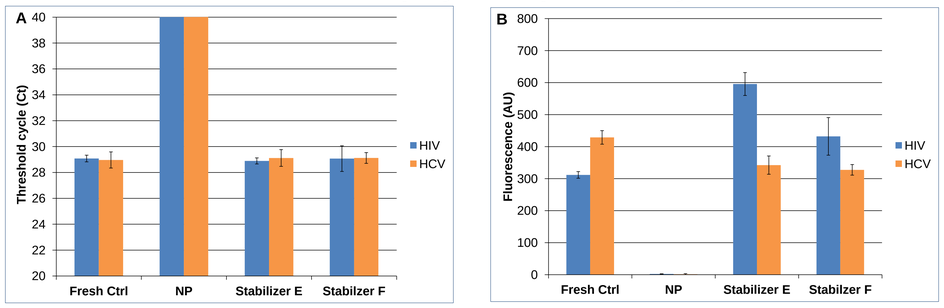

Figure 7: HIV/HCV RT-PCR reactions from dry stabilized HawkZ05 Fast One-step RT-PCR Lyo kit containing enzyme, buffer, dNTPs, and primers & probes. HawkZ05 Fast One-step RT-PCR Lyo mix with primers and probes was stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with RNA template and water. Fresh represents the positive control, NP represents Non Protected control, and Stabilizer E and F 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).

Complete *C. difficile* PCR assay stabilization containing GoTaq

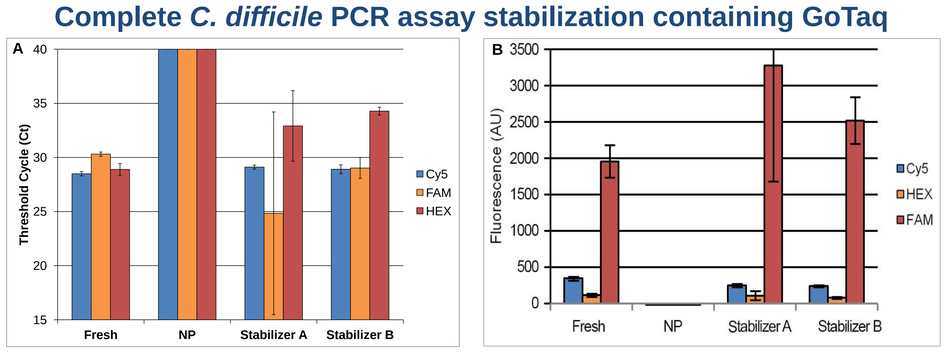

Figure 8: *C. difficile* PCR reactions from dry stabilized GoTaq, PCR buffer, dNTPs, primers and probes. A complete PCR assay for *C. diff* was stabilized and stored for 2 months at 45°C. Following storage, mixes were rehydrated with DNA template and water. **(A)** Quantification cycles of stabilized duplex *C. diff* DNA reactions. 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. Cts of 45 were assigned to NP that failed to amplify.
(B) Endpoint florescence of stabilized duplex *C. diff* DNA reactions following rehydration after 2 months stored at 45°C in dried format.

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 vacuum-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 in Figure 5 shows that real-time RT-PCR reactions using stabilized HawkZ05 DNA Polymerase and nucleotides retain their efficiency to amplify HIV/HCV target RNA after being vacuum-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. Figure 6 shows that similar positive performance can be achieved when stabilizing HawkZ05 DNA polymerase in the presence of primers, probes, and nucleotides. Figure 7 shows that a complete RT-PCR mastermix using the HawkZ05 Fast One-step RT-PCR Lyo kit can also be stabilized.

The data presented in Figure 8 shows that a real-time PCR assay for *C. diff* detection retains its efficiency to amplify *C. diff* DNA after being vacuum-dried in the presence of a PCRstable® stabilizers. Both Ct values and fluorescence are comparable to a fresh positive control.

The data presented demonstrates that RT-PCR and 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.