Development and verification of synthetic RNA controls for determination of Influenza virus load

Maria Mayda, Matt Boley, Shamaila Ashraf, Kurt Langenbach
American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110

ATCC® | THE ESSENTIALS OF LIFE SCIENCE RESEARCH GLOBALLY DELIVERED™

Introduction

Influenza is one of the most significant causes of acute respiratory infection worldwide. Rapid diagnostic tests for highly contagious pathogens, such as Influenza, are essential for decreasing the public health impact of emerging infectious diseases and bioterrorism agents. However, these tests require positive controls that are not always readily available. Consequently, if worldwide public health laboratories are unable to meet the costly regulations required for the import, transfer, and safe use of pathogens used as controls, then critical diagnostic, surveillance, and epidemiological information could be missed.

The use of *in vitro* synthesized viral RNA as a control would provide essential equivalency standards that would be accessible to any laboratory performing quantitative RT-PCR tests. Synthetic RNA controls are particularly useful for laboratories which lack appropriate biosafety containment facilities for propagating a particular pathogenic virus, or have difficulty gaining access to the organism in question due to international tightening of both import and export controls.

Our present work describes the development, use, and evaluation of two stable synthetic RNA controls for influenza A and B, which can be used in place of contagious and unstable RNA in quantitative molecular assays.

Materials and Methods

DNA constructs and preparation of RNA standards

A 1 kb insert containing the target amplicons from segment 7 of Influenza A virus and segment 8 of influenza B virus was cloned, linearized, and transcribed *in vitro*. The transcribed RNA was analyzed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.), and the concentration was quantified using the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen™).

Genomic viral RNA

The genomic RNA preparations used in this study were obtained from BEI Resources: Influenza A virus (NR-2763, NR-2773, NR-2775, NR-10043, NR-10046); Influenza B virus (NR-3178 , NR-3180 , NR-3182 , NR-10047, NR-10048).

Quantitative real-time PCR (qRT-PCR)

qRT-PCR assays were performed using the CDC Universal Influenza primers and probe (Biosearch Technologies), and the CFX96™ system (Bio-Rad Laboratories). The sensitivity of the assay was measured using a set of 10-fold serially diluted RNA synthetic controls with a dynamic range between 10^{-1} and 10^{-9} copies (data not shown).

Droplet Digital™ PCR (ddPCR™)

Detection and quantitation by ddPCR (Bio-Rad Laboratories) was performed using the same primers and probes used in the qRT-PCR assays. ddPCR assays were carried out according to the manufacturer's instructions using Bio-Rad reagents.

Accelerated stability assessment

Aliquots of the synthesized viral RNA were dispensed into 1.5-mL Eppendorf tubes containing commercially available stabilizers, and dried using a SAVANT ISS110 SpeedVac® Concentrator (Thermo Scientific). All dried samples were stored at 50ºC for 8 and 16 weeks. Samples were rehydrated with RNase-free water. To evaluate the functional integrity, two qRT-PCR assays were used for amplification of small (100 bp) and large (1 kb) RNA segments. The Agilent 2100 Bioanalyzer was used to measure the quality of the RNA.

Results

Comparison of qRT-PCR vs. ddPCR results using the quantitative standard

The relationship between qRT-PCR and ddPCR was assessed using two quantitative in-house standards. The concentration of each standard was run in triplicate, and quantitative correlation was observed between methods (Figure 1).

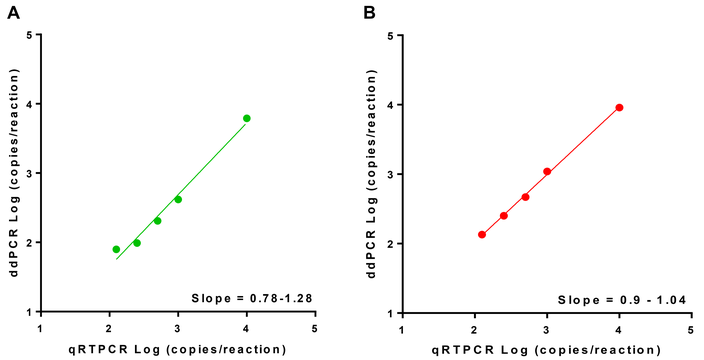

Figure 1: QRT-PCR vs. ddPCR performed on serial dilutions of *in vitro* transcribed RNA standards. A. Influenza A virus. B. Influenza B virus

Comparison of viral load by ddPCR and qRT-PCR

To demonstrate the equivalence of estimating RNA by ddPCR and qRT-PCR, RNA samples were tested in triplicate on three separate qRTPCR runs, and 8 replicates for ddRTPCR. For qRT-PCR, the viral load was calculated by extrapolating the Cq to the standard curve. For ddPCR, the viral load was calculated directly without the use of a standard curve. The mean was summarized for each method (Table 1). A t-test was applied to 8 data points, demonstrating close agreement between the two systems for 8 vRNAs.

Table 1. Real Time RT-PCR and ddPCR detection and quantitation of 10 influenza RNA .

	BEI Resources Catalog No.	N (replicate size)	ddPCR LOG (copies/µL)	qRT-PCR LOG (copies/µL)	P-value
Influenza A	NR-10043	8	2×10^4	3×10^4	0.34
	NR-10046	8	1×10^4	4×10^4	0.16
	NR-2763	8	3×10^3	1×10^4	0.02*
	NR-2773	8	1×10^4	3×10^4	0.19
	NR-2775	8	6×10^3	4×10^4	0.20
Influenza B	NR-10047	8	2×10^4	2×10^5	0.04*
	NR-10048	8	8×10^3	1×10^5	0.05
	NR-3178	8	1×10^5	1×10^6	0.11
	NR-3180	8	1×10^5	8×10^5	0.22
	NR-3182	8	3×10^5	1×10^6	0.23

*Statistically significant

Stability assessment

After 8 and 16 weeks of storage at 50ºC (which is equivalent to 480 years of storage at the desired storage temperature of -80ºC) RNA quality and quantity were compared to -80ºC frozen controls. Quality was confirmed using the Agilent 2100 Bioanalyzer. As compared to pre-stored RNA samples, the frozen and dried samples demonstrated similar RNA Integrity at time 0. However, after 6 weeks at 50ºC, samples without stabilizers degraded (Fig 2A).

The qRT-PCR amplification plot using a small amplicon (100bp) demonstrated comparable amounts of RNA between the frozen (-80ºC) and the 50ºC samples stored in the presence or absence of either stabilizer (Fig 2 B). However, the use of an alternate set of primers that amplify a large amplicon (1kb) clear discernment between RNA stored in presence or absence of stabilizers (Fig 2C).

Our stability data, using *in vitro* transcribed Influenza RNA, demonstrated that the two stabilizers were able to maintain the integrity of RNA stored under temperature stress conditions (50ºC for 16 weeks) with no apparent loss of sample quality as measured by the Agilent 2100 Bioanalyzer and quantitated by qRT-PCR.

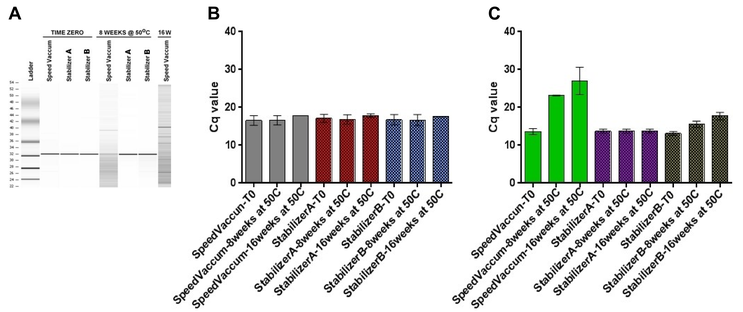

Figure 2: Synthetic Influenza RNA standard stored under accelerated storage conditions (50ºC). **A)** Agilent Bioanalyzer, **B)** qRT-PCR with small amplicon, and **C)** qRT-PCR with large amplicon.

Conclusions and Recommendations

- Stable synthetic RNA controls can be used for qRT-PCR-based assay development and for generation of standard curves for quantification of viral load.
- The use of ddPCR provides accurate and precise quantification of reference material.
- The use of RNA stabilizers offers a unique opportunity for researchers to have access to stable RNA controls as well as potential reductions in the cost options for transporting RNA, especially for international shipments which can require more stringent cold chain management procedures.
- Our approach combining the *in vitro* transcription of RNA, the use of an RNA stabilizer, and ddPCR for absolute quantification, provides unique, well characterized reference materials for the quantification of Influenza A and B.

Disclaimers

 The following reagents were obtained through BEI Resources, NIAID, NIH: Genomic RNA from Influenza A Virus: NR-2763, NR-10043, NR-10046, NR-2773, NR-2775. Genomic RNA from Influenza B Virus: NR-10047, NR-10048, NR-3180, NR-3182