Use of lyophilization to develop a rapid and easy-to-use multiplex real-time PCR assay

Key benefits

- **Multiplex assay**—detection of up to 4 markers simultaneously helps save time and reagents
- **Lyophilized with master mix**—stabilized format allows ambient shipping and room temperature storage
- **Broad instrument compatibility**—suitable for most Applied Biosystems™ QuantStudio™ and 7500 Real-Time PCR Systems
- **Easy to use**—it's simple; just add your samples

Introduction

Singleplex real-time PCR with Applied Biosystems™ TaqMan® Assays has been widely used for analyzing samples for viral nucleic acids [1,2]. However, the limited amounts of nucleic acid obtained from these specimens can make analysis of all relevant targets difficult. Multiplex methods can help resolve this issue by simultaneously detecting multiple infectious agents in a single clinical specimen. A multiplex TaqMan Assay is designed to have multiple probes conjugated to different fluorescent dyes in a single PCR reaction, increasing the amount of information that can be obtained from one sample [3]. For added convenience, lyophilization of TaqMan master mixes provides room temperature storage and increased shelf life while maintaining equivalent sensitivity in multiplex reactions. The potential savings in time and materials make multiplexing of TaqMan Assays an attractive solution for pathogen detection.

Zika virus is a single-stranded RNA arbovirus that is spread to humans primarily through the bite of an infected mosquito. Infected individuals may have fever, rashes, joint pain, and conjunctivitis. A causal link has also been established between Zika virus and birth defects [4]. Countries with local transmission of Zika virus often experience transmission of dengue and chikungunya viruses, which can cause similar symptoms.

These three viruses can be simultaneously detected in serum by analyzing research samples for the presence of viral nucleic acids or virus-specific antibodies. Since serological cross-reactivity is strong between the Zika and dengue viruses, detection of viral RNA using reverse-transcription PCR (RT-PCR) is highly recommended [5,6].

Here we describe how we developed a 4-plex TaqMan Assay [7] that simultaneously detects Zika, chikungunya, and pan-dengue viral RNA, as well as the human peptidylprolyl isomerase A (*PPIA*) gene as an extraction and endogenous control. Lyophilized assays are used to enable ambient shipping and room temperature storage, and they deliver performance similar to the liquid singleplex assays.

Materials and methods

RNA extraction

Human RNA was purified from normal uninfected serum using the Applied Biosystems™ MagMAX™ Pathogen RNA/DNA Kit (Cat. No. 4462359).

TaqMan multiplex assay design

Since the same mosquito transmits Zika, chikungunya, and pan-dengue viruses, a multiplex TaqMan Assay was designed using published sequences to identify RNA from the three viruses in a one-tube reaction. The TaqMan Assays that identify each target (Table 1) are lyophilized along with Applied Biosystems™ Mustang Purple™ passive reference dye and a 1-step RT-qPCR master mix in an 8-well strip format. Analyses of individual viral sequences using real-time PCR have been reported previously [4-8].

Table 1. TaqMan Assays used in the multiplex reaction.

Target	Probe label
Zika	FAM™ dye
Chikungunya	ABY™ dye
Pan-dengue	VIC™ dye
PPIA exon 5	JUN™ dye

Real-time RT-PCR

RNA samples (25 µL) were added to the lyophilized assay, and RT-PCR was conducted on a QuantStudio 7 Flex Real-Time PCR System using standard cycling conditions [9] (Table 2). To simulate an RNA sample that would be extracted from virus-infected blood, and to serve as a positive control for the RT-PCR reaction, the purified human serum RNA was spiked with *in vitro*–transcribed RNA of the three viruses. For the determination of the lower limit of detection (LOD) of the assay, serial dilutions were made from RNA extracted from cultured isolates of the three viruses.

Table 2. Cycling conditions for RT-qPCR.

Step	Cycling condition	
Reverse transcription	Hold	50°C for 20 min
Polymerase activation	Hold	95°C for 2 min
PCR amplification	40 cycles	95°C for 15 sec, 60°C for 1 min

Results

Real-time RT-PCR on the serum/virus RNA mixture shows that all three viral targets and the endogenous control *PPIA* are amplified reliably in the same reaction (Figure 1).

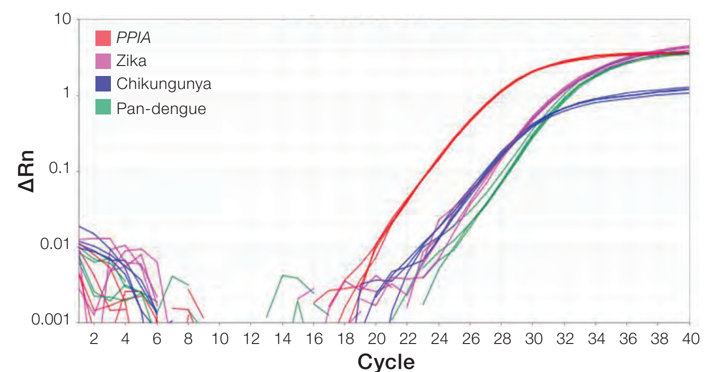
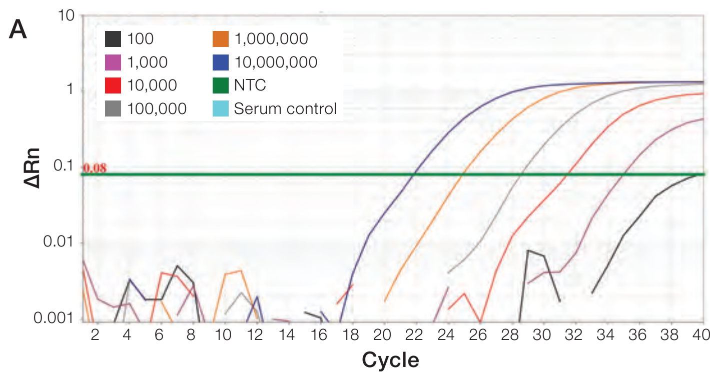
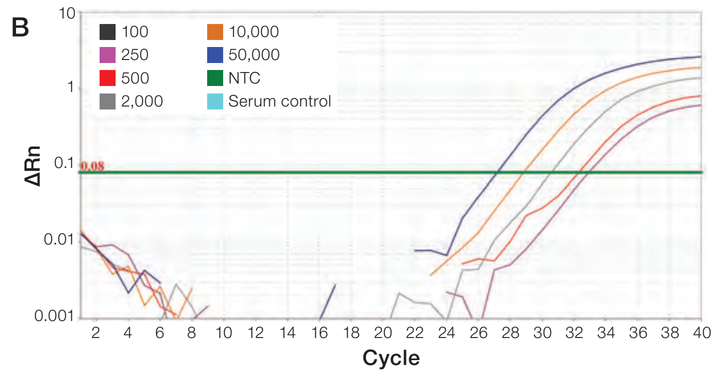


Figure 1. Positive control results using synthetic RNA and the *PPIA* gene as an endogenous control.

A series of six dilutions was prepared from extracted viral RNA of each target, down to 100 copies/mL. Two negative control samples were used: water (no-template control) and RNA extracted from normal human serum. Amplification curves for the dilutions of each target are shown in Figure 2. Control samples did not have any amplification signals.

We also compared LOD data of our triplex assay with published data (Table 3). LOD was determined as the last dilution where at least 19 of 20 replicates were positive. The multiplex assay demonstrated specificity for the intended targets (data not shown), similar to previously described data for a triplex real-time RT-PCR assay [10].





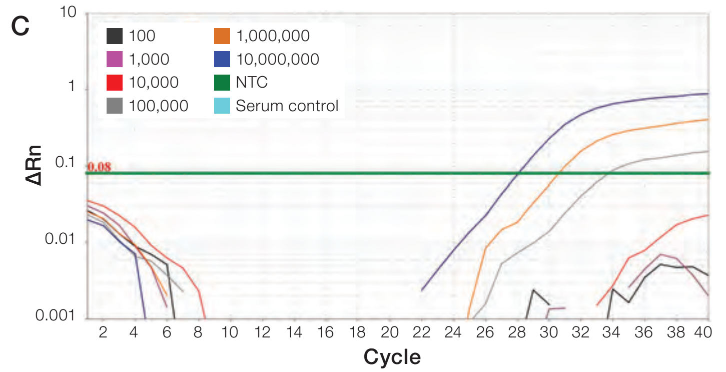


Figure 2. Amplification plots of serially diluted viral RNA. (A) Dengue virus serotype 3, **(B)** Zika virus (Asian strain), and **(C)** chikungunya virus were detected using the multiplex TaqMan Assay.

Table 3. LOD of each assay in the multiplex reactions.

	LOD in copies per mL of serum sample	
Virus	**Applied Biosystems™ TaqMan® Zika Virus Triplex Kit**	**Published triplex real-time RT-PCR assay**
Zika (Asian strain)	250	15,400
Chikungunya	100,000	399,000
Dengue serotype 1	2,500	21,500
Dengue serotype 2	3,500	17,200
Dengue serotype 3	2,000	16,600
Dengue serotype 4	850	13,600

applied biosystems

Conclusions

This study demonstrates that lyophilized multiplex TaqMan Assays, such as the TaqMan Zika Virus Triplex Kit or other custom assays, can be easily developed and optimized for simple, accurate, and rapid detection of viral RNA. These multiplexed assays offer:

- Results similar to those obtained using singleplex TaqMan Assays
- A simple protocol with about 5 minutes of hands-on time
- Easy transportation and storage along with prolonged shelf life enabled by the lyophilized format

References

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9. TaqMan multiplex qPCR: Accurate, sensitive, and as efficient as traditional qPCR. Application note available at thermofisher.com (Pub. No. CO28780 0514).
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Ordering information

Product	Quantity	Cat. No.
TaqMan Zika Virus Triplex Kit	0.2 mL, 12 x 8-well strip tubes	A31746
	0.1 mL, 12 x 8-well strip tubes	A31747
QuantStudio 5 Real-Time PCR System	1 instrument	A28138
QuantStudio 7 Flex Real-Time PCR System	1 instrument	4485690
7500 Fast Dx Real-Time PCR Instrument	1 instrument	4406984
MagMAX Pathogen RNA/DNA Kit	480 preps	4462359

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