A Multiplex qPCR Assay for Zika, Dengue, and Chikungunya Viruses (ZDC) to Support On-going Vaccine and Drug Efficacy Studies

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Introduction

- The global spread and complications associated with Zika virus (ZKV), dengue virus (DENV), and chikungunya virus (CHIKV) have made these Flaviviruses a major public health concern. DENV can progress to severe viral hemorrhagic fever, ZKV is associated with congenital and neurological anomalies which require an early diagnosis during pregnancy, while CHIKV often leads to debilitating polyarthritis.
- The approval, in 1986, of a series of recombinant hepatitis B vaccines was a landmark both in the growth of biotechnology and in the development of a new age of cutting edge vaccines. These innovative recombinant approaches to vaccine development require innovative bioanalytical tools to assure the quality, safety and efficacy of recombinant vaccines.
- We have developed a one-step RT-PCR assay for the multiplex detection of Zika, dengue (DENV1, DENV2, DENV3, and DENV4), and chikungunya viral RNA by real-time PCR. This assay can be utilized to support viral RNA quantification in the following areas of research and development:
  - **Vaccine formulation & potency -** To determine quality and dose of the vaccine product. Also the potency of the vaccine product as part of an infectivity PCR assay.
  - **Preclinical safety -** To measure in animal serum and tissues to support pre-clinical toxicity studies
  - **Biodistribution studies:** To determine presence and persistence of the vaccine product in various tissues
  - **Clinical development -** To determine the infectious status of individuals taking part in clinical trials of novel vaccines
  - **Clinical development -** To assess viremia status
  - **Clinical Development -** As an endpoint measurement of the virus neutralization assay

Method

Inactivated virus controls (ZKV and CHIKV, Zeptometrix Corporation), spiked into either kit buffer or human urine or plasma, were processed for total viral nucleic acid using Promega Maxwell® RSC automated system. Kit positive control RNA, containing sequence of all three viruses, was extracted alongside all samples. An internal control RNA, which does not contain known human or viral sequence, was added to all samples as a processing control. The Bio-Rad ZDC Multiplex RT-PCR Assay was used to detect viral RNAs on an AB7500 real-time PCR instrument (Applied Biosystems®) using the following fluorophores: ZKV-FAM, CHIKV- HEX, DENV-TexasRed, Internal Control-Cy5. Reaction mix consisted of iTaqTM Universal Probes One-Step Reaction Mix, iScriptTM Reverse Transcriptase, ZDC Multiplex PCR Assay Mix, viral nucleic acid template, and water. Thermocycling conditions were as follows: Stage 1 at 50°C for 15 minutes, Stage 2 at 94°C for 2 minutes, and 45 cycles of Stage 3 at 94°C for 15 seconds, followed by 55°C for 40 seconds, then 68°C for 30 seconds.

Figure 1: 200 µL of each virus control (ZKV^{hi}, ZKV^{lo}, or CHIKV) or kit positive control was processed for viral nucleic acid which was then serially diluted 1:5

Assay performance over a range of input concentrations

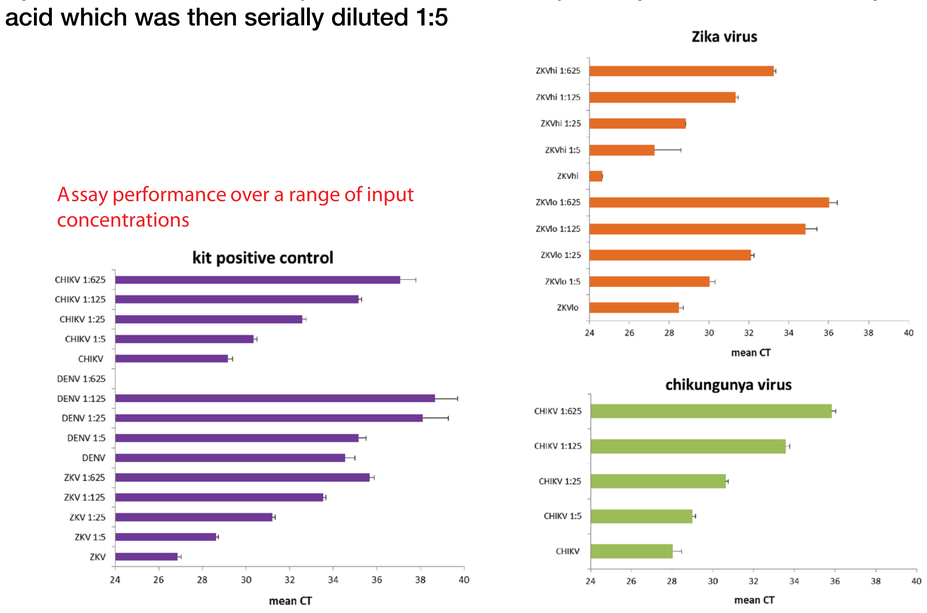


Figure 2: Various volumes (100, 25, 6.25, or 1.6 µL) of virus controls (ZKV, CHIKV, or ZKV/CHIKV mixed) were spiked into either kit Buffer T or pooled human urine

Spike -volume dependent amplification
Independent of matrix effects
Independent of presence of other virus

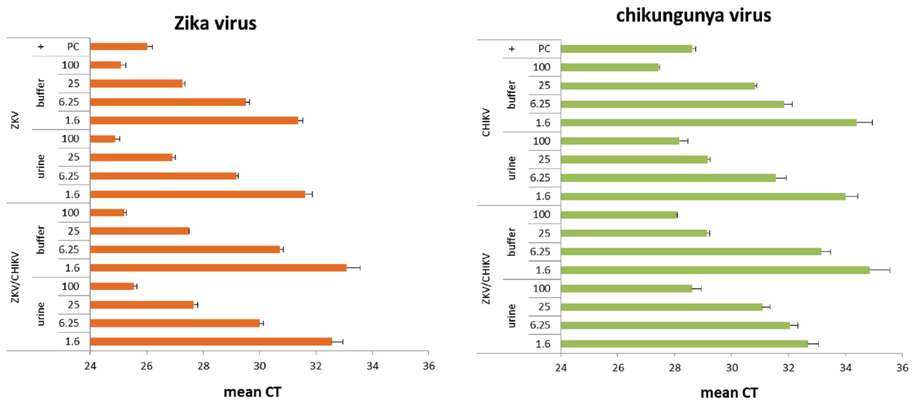


Figure 3: Various volumes (100, 25, 6.25, or 1.6 µL) of virus controls (ZKV, CHIKV, or ZKV/CHIKV mixed) were spiked into either kit Buffer T or pooled human plasma

Spike -volume dependent amplification
Independent of matrix effects
Independent of presence of other virus

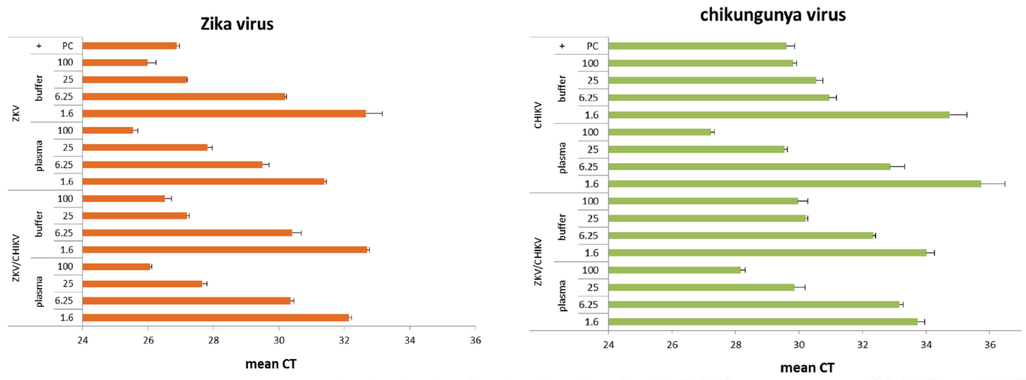
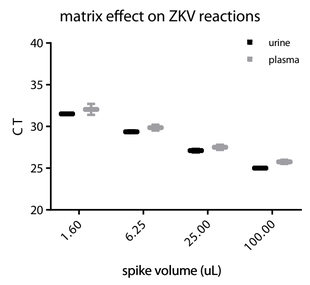


Figure 4 and Table 1: Various volumes (100, 25, 6.25, or 1.6 µL) of ZKV or CHIKV virus controls were spiked into either kit Buffer T or pooled human urine or plasma



Spike -volume dependent amplification

Independent of matrix effects

matrix effect on ZKV reactions

spike vol (uL)	mean CT urine	mean CT buffer	%CV	mean CT plasma	mean CT buffer	%CV
1.6	31.6	31.4	0.58	31.4	32.7	2.80
6.25	29.2	29.5	0.81	29.5	30.2	1.62
25	26.9	27.3	0.96	27.8	27.2	1.69
100	24.9	25.1	0.61	25.5	26.0	1.24

matrix effect on CHIKV reactions

spike vol (uL)	mean CT urine	mean CT buffer	%CV	mean CT plasma	mean CT buffer	%CV
1.6	34.0	34.4	0.81	35.7	34.7	2.00
6.25	31.6	31.8	0.63	32.9	31.0	4.25
25	29.2	30.8	3.91	29.5	30.5	2.37
100	28.2	27.4	1.88	27.2	29.8	6.39

Figure 5 and Table 2: Various volumes (100, 25, 6.25, or 1.6 µL) of virus controls (ZKV, CHIKV, or ZKV/CHIKV mixed) were spiked into either kit Buffer T or pooled human urine or plasma

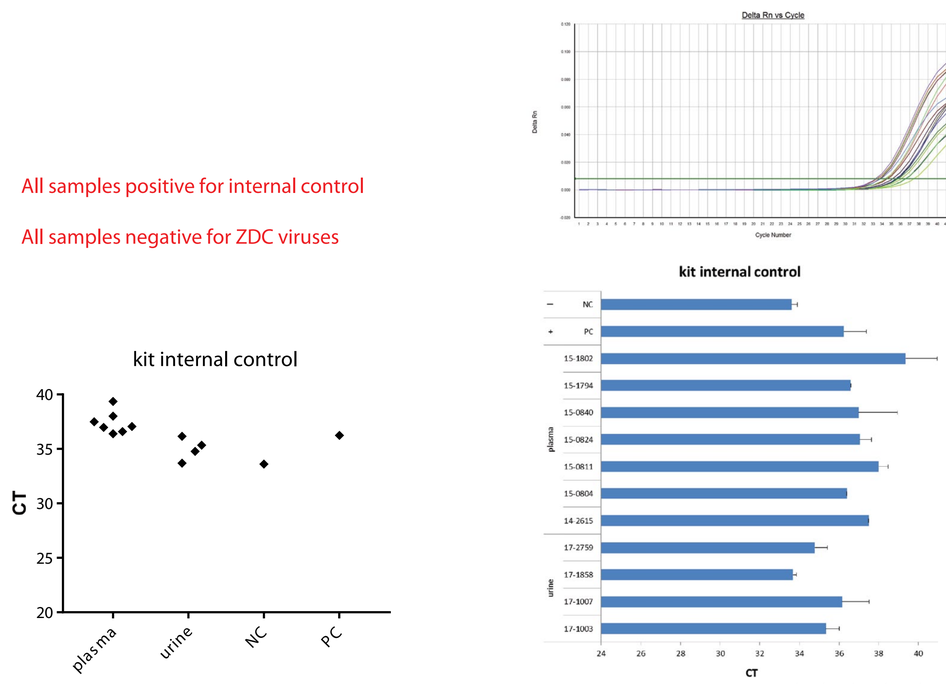
CHIKV effect on ZKV reactions

spike vol (uL)	urine mean CT ZKV	urine mean CT ZKV/CHIKV	urine %CV	plasma mean CT ZKV	plasma mean CT ZKV/CHIKV	plasma %CV	buffer mean CT ZKV	buffer mean CT ZKV/CHIKV	buffer %CV
1.6	31.6	32.6	2.06	31.4	32.1	1.67	32.7	32.7	0.09
6.25	29.2	30.0	1.98	29.5	30.4	2.01	30.2	30.4	0.51
25	26.9	27.7	1.97	27.8	27.7	0.42	27.2	27.2	0.05
100	24.9	25.5	1.88	25.5	26.1	1.44	26.0	26.5	1.43

ZKV effect on CHIKV reactions

spike vol (uL)	urine mean CT CHIKV	urine mean CT ZKV/CHIKV	urine %CV	plasma mean CT CHIKV	plasma mean CT ZKV/CHIKV	plasma %CV	buffer mean CT CHIKV	buffer mean CT ZKV/CHIKV	buffer %CV
1.6	34.0	32.7	2.78	35.7	33.7	4.06	34.7	34.0	1.50
6.25	31.6	32.1	1.09	32.9	33.2	0.62	31.0	32.3	3.07
25	29.2	31.1	4.53	29.5	29.9	0.76	30.5	30.2	0.75
100	28.2	28.6	1.09	27.2	28.2	2.40	29.8	30.0	0.40

Figure 6: Individual lots of human urine or plasma were spiked with kit internal control and processed for viral nucleic acid



Spike -volume dependent amplification

Independent of presence of other virus

All samples positive for internal control

All samples negative for ZDC viruses

Conclusion and Future Work

- For innovative therapies such as vaccine based, measurement of nucleic acids requires an innovative bioanalytical tool that can be validated in a regulatory compliant manner.
- We have successfully validated a one-step RT- PCR assay for the multiplex detection of Zika, dengue, and chikungunya viral RNA by real-time PCR. The assay was found to be sensitive, specific, and reliable for the semi-quantitative measurement of ZKV, DENV, and CHIKV RNA in nucleic acids extracted from a wide range of sample types.
- In the future, we intend to utilize this method for the measurement of neutralization endpoint to support the virus neutralization assay. Virus neutralization assay is considered to be the most specific assay for measurement of anti-virus antibodies in clinical trials to support efficacy.
- Virus neutralization assay is most commonly performed in the form of a plaque reduction neutralization test (PRNT). The assay measures the production of antibodies that functionally inhibit viral infection which is key to demonstrating efficacy of vaccines in a clinical trial. However, PRNT method is expensive, labor intensive, requires cell-culture facilities, and takes up to 10 days to generate results. We anticipate that by using this RT-PCR method, we can reduce the cost significantly as this method is automation friendly compared to measurement of plaque reduction by PRNT. It will also allow us to reduce the turnaround time to 3 days, **allowing for streamlined assessment of vaccine efficacy in clinical trials.**