# NOVEL MULTIPLEX METHOD FOR DETECTION OF ZAIRE EBOLAVIRUS(ZEBOV) IN BLOOD AND ORAL FLUIDS BY REAL TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (rRTPCR)USING A PORTABLE POINT OF CARE (POC) PLATFORM

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## ABSTRACT

Background: Zaire ebolavirus (ZEBOV) is a member of the order Mononegavirales, family Filoviridae. It assembles heterogeneous, filamentous, enveloped virus particles containing a negative sense, single stranded RNA ~ 19 kb genome with 7 distinct genes (Fig 1) packaged within a helical nucleocapsid. Fatality rates for ebolaviruses range from 40% to 90%. In the ongoing epidemic in western Africa, Zaire strain has

resulted in over 23,500 cases with over 14,000 laboratory confirmed cases and over 9,500 deaths as per CDC. With the objective of countering this current and imminent threat we have designed and developed a dry format multiplex reverse transcription polymerase chain reaction assay designed for field use. In this paper we present data showing optimization of this assay for use with portable TCOR 8™ platform using simple sample collection and sample processing devices to detect ZEBOV in whole blood and oral fluid samples. The objective of the study is to develop an integrated system that includes assays and a portable platform suitable for point of care settings and low resource settings.

Methods: We have designed a sensitive and specific multiplexed rRTPCR assay for rapid detection of ZEBOV using the published sequence. This ZEBOV triplex assay covers three specific targets; two in nucleoprotein (NP) gene and one in polymerase (L) gene of ZEBOV (Fig 3a). These targets combined in one assay were designed to work in concert to provide broad detection of the viral RNA, mitigating the potential for the virus to escape detection through viral mutation. RNase P internal control (Fig 3b) for monitoring the functionality of template and to monitor inhibition of assay is also added in this multiplex. This assay design was evaluated using extracted RNA from ZEBOV, Mayinga strain (BEI resources, Cat # NR31806).

Multiple sets of primers and probes were selected based on this study and further evaluation of selected sets was carried out on the lab based platform ABI 7500 and POC platform TCOR 8™. Sample preparation was optimized using gamma irradiated ZEBOV, Mayinga strain (BEI, Cat # NR31807) spiked in whole blood and oral fluid samples.

Results: Extracted ZEBOV RNA from BEI resources was diluted in assay buffer and tested on the TCOR 8 platform. Comparison of virus spiked in assay buffer or biological matrices showed that the assay is more sensitive in assay buffer. This was attributed to destruction of naked RNA template in human blood and oral fluid matrices. RNase P assay was optimized as an internal control using both oral fluid and whole blood matrices to give Ct value of about 30 on TCOR 8 platform. Further, the gamma irradiated virus was diluted 10 fold serially from 1000 fold dilutions to $10^8$ fold dilutions in whole blood, oral fluids and assay buffer. The spiked samples were tested on both ABI 7500 and TCOR 8 platforms. The comparison shows that virus could be detected on both platforms with similar sensitivity. Further optimization of the assay is being carried out.

Conclusions: This novel near patient system for detection of ZEBOV using the dried down reagents and the portable point of care platform TCOR 8 are an optimal choice for efficient use in remote and low resource areas. This integrated system would potentially eliminate transportation of infected samples to a centralized laboratory.



**Fig 1**: Graphic representation of 7 genes in ebolavirus genome.

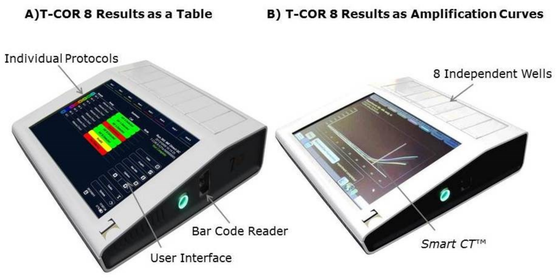
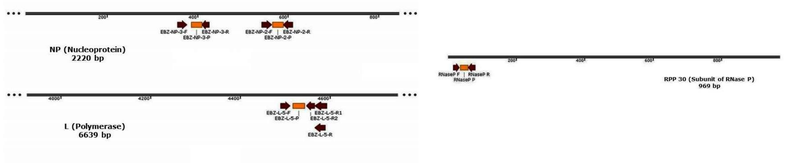



**FIG 2 :** Portable Platform T-COR 8 for Point of Care Diagnosis




3a) Primers and probes for ZEBOV

3b) Primers and probe for RNase P

**FIG 3:** Primers and Probes for a) ZEBOV and b) RNase P

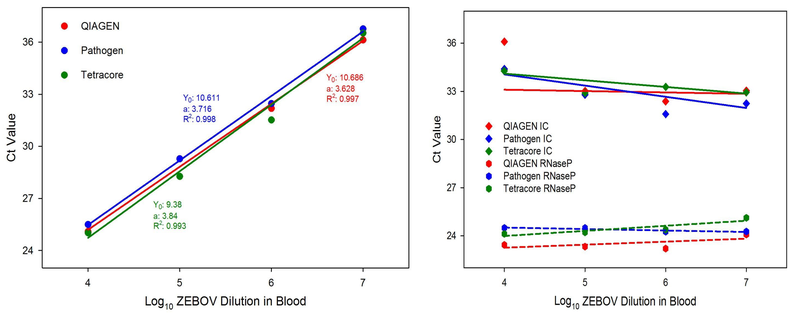



**FIG 4:** Comparison of different extraction methods for detection of ZEBOV in blood

| Dilution | Day 1 | | | Day 2 | | | Day 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | ZEBOV | RNase P | IC | ZEBOV | RNase P | IC | ZEBOV | RNase P | IC |
| 1.00E+04 | 25.0 | 24.1 | 34.3 | 25.1 | 24.2 | 34.2 | 26.1 | 24.2 | 33.2 |
| 1.00E+05 | 28.3 | 24.2 | 32.9 | 28.2 | 24.2 | 32.5 | 29.2 | 24.4 | 33.6 |
| 1.00E+06 | 31.5 | 24.4 | 33.3 | 31.5 | 24.5 | 33.0 | 32.3 | 24.8 | 33.6 |
| 1.00E+07 | 36.5 | 25.1 | 32.9 | 37.2 | 24.9 | 32.7 | 36.3 | 24.5 | 33.4 |

**Table 1:** Day to day repeatability with Tetracore extraction method in blood

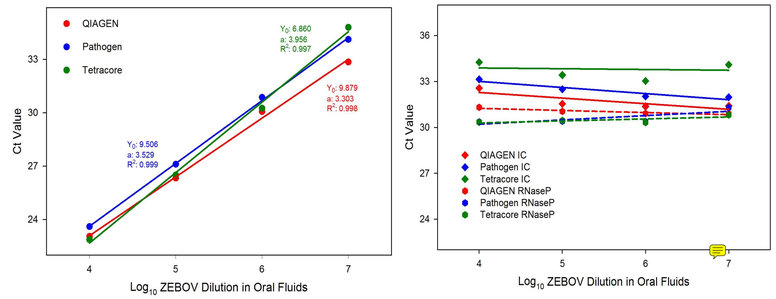



**FIG 5:** Comparison of different extraction methods for detection of ZEBOV in oral fluids

| Dilution | ZEBOV | RNase P | IC |
|---|---|---|---|
| 1.00E+09 | ND | 33.7 | 34.2 |
| 1.00E+08 | 38.9 | 31.6 | 34.7 |
| 5.00E+07 | 36.9 | 32.3 | 36.3 |
| 1.00E+07 | 35.2 | 32.4 | 32.7 |
| 1.00E+06 | 31.7 | 31.7 | 36.3 |
| 1.00E+05 | 27.5 | 31.6 | 33.4 |

**Table 2:** Extended Titration in oral fluids with Tetracore extraction method

## RESULTS

Gamma-irradiated Zaire ebolavirus, Mayinga was obtained from BEI resources where it was prepared from infected Vero E6 cell pellets that were resuspended in 50 mM sodium borate and 120 mM sodium chloride (pH 9) containing 1% Triton X-100, gamma-irradiated ($5 \times 10^6$ RADs) on dry ice, and sonicated. This was diluted serially in blood from 1e4 to 1e7 fold and RNA extraction methods were optimized for use in field. Comparison of Qiagen RNA extraction kit, Pathogen extraction kit and simple Tetracore method of extraction is shown in Figures 4 and 5 for blood and oral fluids respectively. Tetracore novel sample preparation method was found to be comparable to Qiagen and Pathogen extraction methods.

Day to day reproducibility was tested for sample preparation in blood using same blood donor and extraction method over 3 days and method was found to be reproducible (Table 1). Individual to individual variability was tested using oral fluids from two different individuals. An extended titration was performed and results indicate that ZEBOV could be detected in oral fluids down to a dilution of 1e8 fold (Table 2).

## CONCLUSIONS

Complex, cumbersome and slow diagnostic tests pose a great challenge to contain the outbreaks on ZEBOV. Molecular tests currently being used also impose additional logistical challenges such as staff expertise, lab instrumentation, a full tube of blood, and high cost which are difficult to meet in resource limited areas. We are working towards developing a point of care method that uses a simple extraction procedure and a device that can be used with relative ease in field. Further validation of our novel platform is ongoing. Our current easy to use POC platform shows a great promise in overcoming the challenges of ZEBOV detection in resource limited and near patient settings.