

DIGITAL DROPLET PCR QUANTIFICATION OF HUMAN T-LYMPHOTROPIC VIRUS TYPE-1 CLADE C IN PERIPHERAL BLOOD AND LUNG INFILTRATES OF INDIGENOUS AUSTRALIAN BRONCHIECTASIS PATIENTS

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INTRODUCTION

Human T-cell Lymphotropic Virus type-1 (HTLV-1) infects an estimated 20 million people worldwide causing a fatal T-cell leukaemia and inflammatory conditions, such as HTLV-1-associated myelopathy, resulting from a dysregulated inflammatory response to the virus. HTLV-1 is highly endemic to remote indigenous communities in Central Australia where infection contributes to racial disparities in morbidity and mortality. HTLV-1 related complications are associated with elevated numbers of T-cells in which the HTLV-1 provirus integrates (HTLV-1 proviral load, PVL). Digital-droplet PCR (ddPCR) is capable of absolute quantification of PVL, offering low variability between assays with few cell numbers. Using ddPCR, we measured HTLV-1 PVLs from buffy coat cells (BCCs) of infected individuals, and bronchoalveolar lavage (BAL) infiltrates from a bronchiectasis patient.

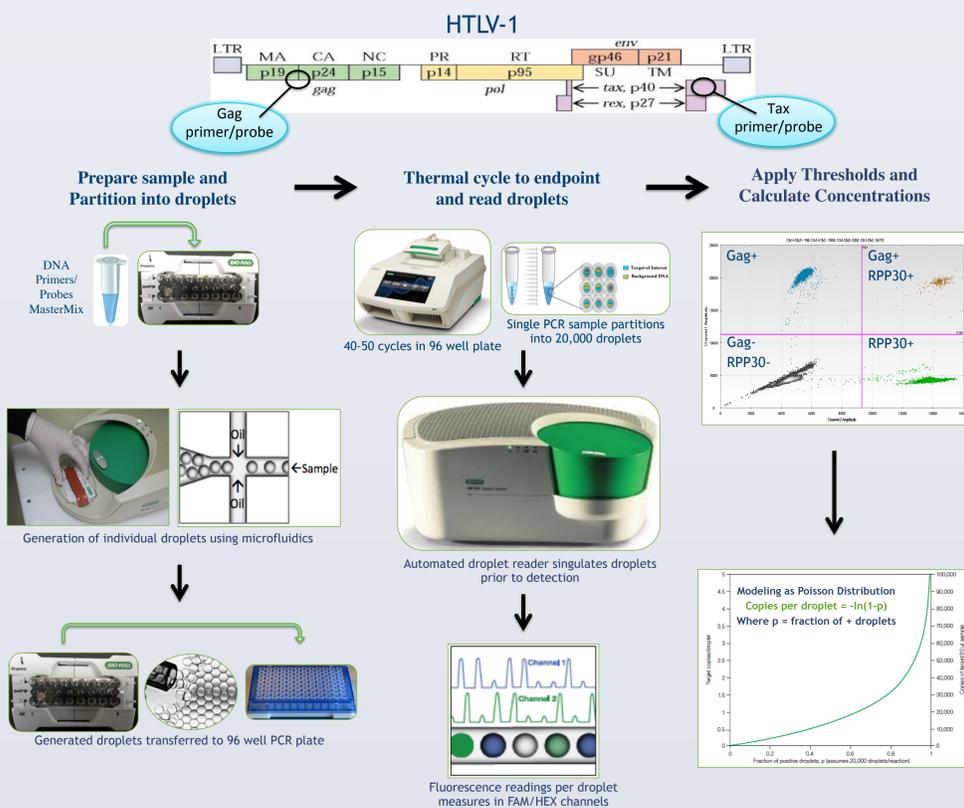
MATERIALS & METHODS

All samples were from Alice Springs Hospital HTLV-1 cohort (30 BCCs, 1 BAL) and obtained following ethics approval (HREC-14-249) and patient consent in first language. Genomic DNA was extracted from BCC and BAL samples using DNA extraction kit (Qiagen) and used for ddPCR PVL quantification.

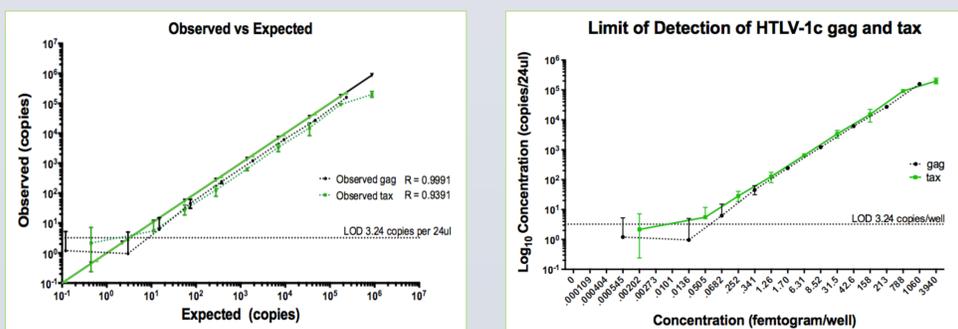
This assay was performed in 40 µl PCR mixture volume consisting of Supermix no dUTPs (Bio-Rad) containing DNA polymerase, 900nM/250nM of each oligonucleotide primer/probe, and 50ng of DNA extracted from peripheral blood or lung infiltrate. Details of HTLV-1 gene amplification were: 10min/95°C followed by 40 cycles of (95°C/30s, 58°C/30s, 72°C/30s). HTLV-1c gag/tax primer sequences and FAM/MGB probes were optimized using temperature gradient amplification.

The sequence of these primers and probes were; Gag-for: 5'CAAATGAAGGACCTACAGGC3'; 3' Gag-rev: 5'TATCTAGCTGCTGGTGATGG3'; Gag-Probe: 5'FAM-ACCATCCGGCTTGAGT-MGBNFQ3'; Tax-for: 5'TCCAGGCCATTATTTGGACAT3'; Tax-rev: 5'CGTGTGAGAGTAGGACTGAG3'; Tax-Probe: 5'FAM-CATGATTTCCGGCCCTTGC-MGBNFQ3'.

Samples were tested in duplicate relative to an internal reference gene standard, ribonuclease protein 30kDa subunit (RPP30) and negative template controls (NTC). QuantaSoft software was used for data analysis. Plasmid constructs with HTLV-1-specific viral sequences were diluted to characterize the distribution of measured droplet events at the upper and lower limits of detection. The sensitivity of the method used is 3 copies of proviral DNA per 10⁶ cells.



ddPCR Limit of Detection (LoD) targeting HTLV-1c gag and tax

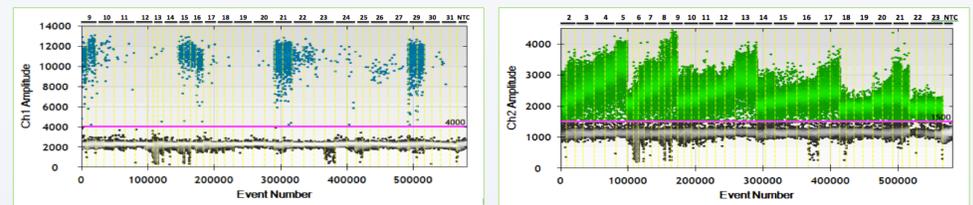


A 1:5 series dilution of plasmid containing HTLV-1c viral targets (pCR11-gag and pCR11-tax) were used to determine the number of HTLV copies. Comparison of the observed number of copies from each target (y-axis) with the expected number of copies (x-axis) provides an estimation of the assay accuracy.

The dilution series show linearity from 3.2 to 1x10⁵ copies/well. Where the data points stray from linearity represents the lower LoD (3.24 copies per well), and the machine fails to provide accurate sample measurement.

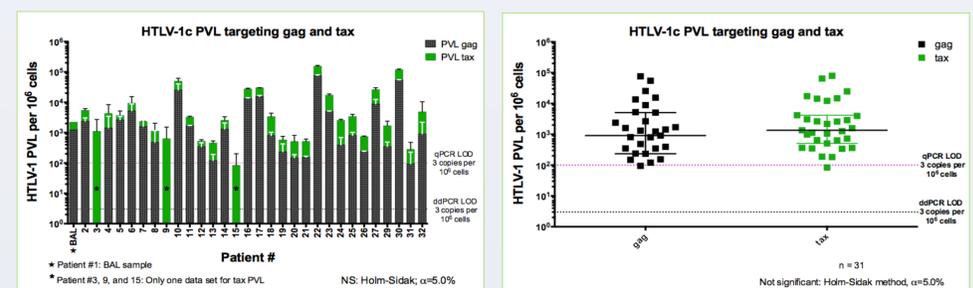
RESULTS

ddPCR analysis of HTLV-1c seropositive patients



ddPCR analysis of HTLV-1c seropositive patients #9-31 displays excellent separation between positive droplets (blue) and negative droplets (black). Two channels were used simultaneously to detect a fluorescent 5' reporter (FAM-HTLV1c, Ch1) or (HEX-RPP30, Ch2). Unlike qPCR, measurement of target DNA does not require a standard curve. The non-template control (NTC) displays an entirely negative droplet population which determines threshold set point. RPP30 is a ubiquitous housekeeping gene found in humans that has been used as an internal reference gene to calculate the PVL.

PVL of HTLV-1c seropositive patients



Proviral Load (PVL) is defined as the number of HTLV-1 copies per 10⁶ cells. Gag and tax-specific viral sequences were measured in duplicate for each patient (except #3,9,15), with RPP30 used as an internal reference gene. LoD using ddPCR was previously determined as 3 copies per 10⁶ cells, and the ddPCR assay is well within the range of detecting the presence of HTLV-1 targets gag and tax. Targeting both HTLV-1-gag and tax resulted in similar mean HTLV-1c PVL (~1000 HTLV+ per 10⁶ cells).

CONCLUSION

The ddPCR assay demonstrates extremely high sensitivity, low assay-variability and the capability to reliably quantify HTLV-1 PVL. The power of ddPCR exhibits unparalleled precision enabling small fold differences in target DNA sourced from cell debris. This technique offers logistic advantages in studying relationships between PVL from HTLV-1 patient's BCCs and BALs.

In this study, variation in PVL between patients was detected, which has been previously described as a result of mutations in target regions or multiple integrations of the proviral genome (Kamihira et al, 2010). PVL variations might be a result of differences between individual host immune response and viral genetics (Niederer and Bangham, 2014).

HTLV-1c PVL of all patient samples were well within the range of detectability of the ddPCR assay. Of the 30 BCCs, the PVL ranged from 143 - 297,800 proviral copies per 10⁶ cells. The BAL sample PVL was 1,110 DNA copies per 10⁶ cells. Using ddPCR to detect HTLV-1 viral targets, the LoD was determined at 3 copies of proviral DNA per 10⁶ cells, which is 30-fold more sensitive than a qPCR LoD assay previously used to measure HTLV-1 from the same patient cohort (Einsiedel et al., 2014). The ddPCR assay is able to measure HTLV-1 PVL at sample amounts lower than the LoD for qPCR, allowing early detection of HTLV-1 seropositive patients.

LITERATURE CITED

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