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Touchdown digital PCR for quantification of difficult amplicons in the HIV Genome

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BACKGROUND:

Digital PCR (dPCR) has long been proposed as an alternative absolute quantification method to current quantitative real-time PCR (qPCR). With recent technological developments, dPCR is now possible at high throughput and at limited costs. As an example, Bio-Rad recently introduced the QX100™ Droplet Digital™ PCR Platform (ddPCR; Fig. 1). DPCR is believed to be a more accurate absolute quantification method compared to qPCR as dPCR does not rely on PCR efficiency for accurate quantification. Consequently, this technique forms a promising tool for HIV research and multiple applications are currently being investigated.

In contrast to qPCR, dPCR does not rely on the kinetics of the PCR reactions. As long as sufficient fluorescent signal is obtained in positive replicates (droplets) then accurate quantification can proceed. This additional advantage of dPCR enables a high flexibility of assay design and cycling conditions.

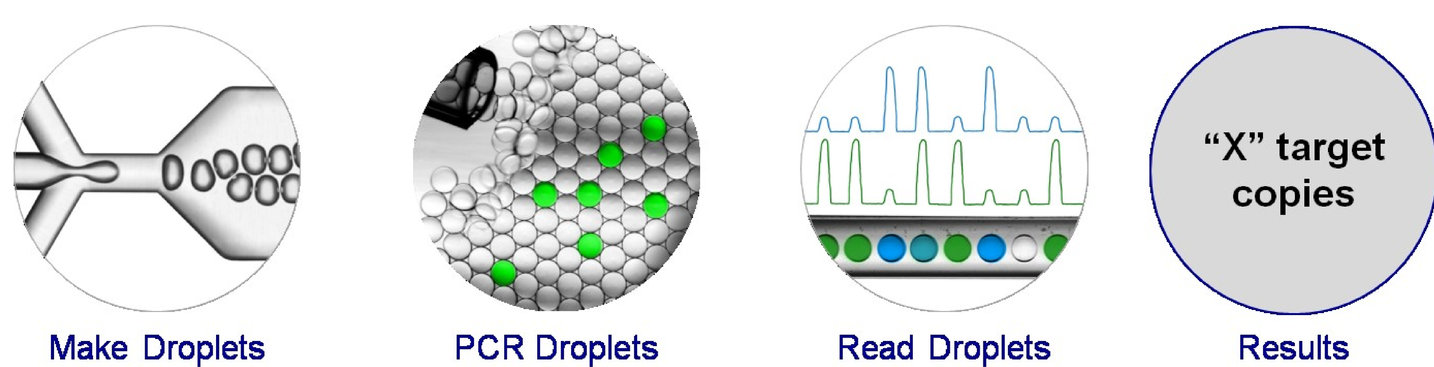
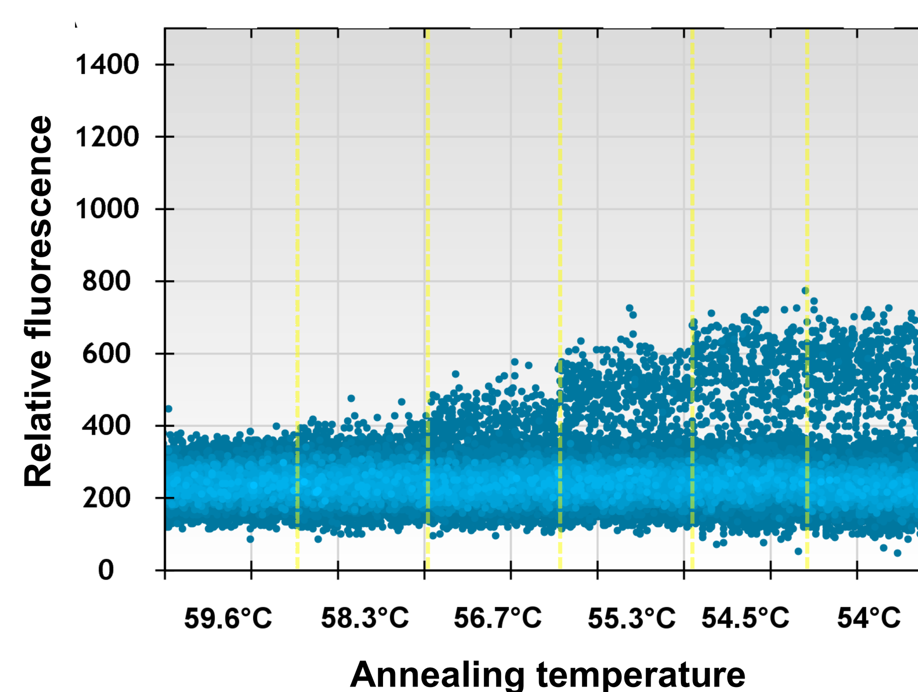


Figure 1: Schematic representaion of the ddPCR workflow. First droplets of PCR mix with primers, probes and sample are made in an oil suspension. These droplets form picoliter scale replicate PCR reactions. Fluorescence is subsequently measured in each droplet by flow cytometery. Finally, Poisson calculation of positive versus negative droplets enables absolute quantification of input DNA without the requirement of a standard curve.

PROBLEM STATEMENT:

Due to the high variability in HIV-1 variants within and between patients the development of assays that accurately detect HIV sequences is complicated. Recently, Van Der Sluis et al. (2013, J Virol Methods 187:94-102) developed a set of primers and probes that specifically target a highly conserved region in the HIV genome of all recorded HIV-1 variants. However, due to the small sequence available for assay design, the probe is characterized by a low melting temperature. Initial validation by a gradient ddPCR with decreasing annealing temperatures revealed that ddPCR for this assay is only possible at temperatures lower then 55°C (Fig 2).

Figure 2: Dot plot output of the gradient ddPCR. Negative droplets have a background fluorescent signal, here between 150 and 400. Positive droplets have higher fluorescence. Absolute quantification is based on the ratio of positive versus negative droplets.
In order to calculate this ratio, a treshold should be set based on the negative population. However, as can be observed on the current plot, the positive population cannot be distinguished unambiguously from the negative droplets, hampering quantification even at temperatures lower than 55°C. We hypothesized that the low positive fluorescence was due to the low melting temperature of the probe hampering efficient hydrolysis



OBJECTIVE:

A strategy was investigated to maintain high reaction specificity, while allowing accumulation of fluorescent signal for quantification by using a two stage touchdown PCR.

METHODS & RESULTS:

Initial ddPCR tests were performed on plasmids containing the HIV-1 derived lab strain NL4-3 sequence. Different touchdown strategies were compared and quantification of plasmids was correlated to quantification by standard ddPCR using primers and probes specific for the NL4-3 sequence (Table 1).

The two stage touchdown protocols (Fig. 3) consisted of a first set of stringent PCR conditions (58°C), allowing specific amplification. Subsequently, the annealing temperature was lowered (50° C) to allow optimal fluorescent accumulation.

Table 1: Primers and probes used for ddPCR detection

name		sequence	gene	Ta °C	nM
HIVPf	forward	GCCTCAATAAAGCTTGCCTTGA	HIV-1	58	200
HIVPr	reverse	GGGCGCCACTGCTAGAGAT	HIV-1	58	200
HIVPpr	probe	GTA[a/t/g]CTAGAGATCCCTCAGA	HIV-1	<55	200
HIVNL4.3f	forward	TGTGTGCCCGTCTGTTGTGT	HIV-1 NL4-3	58	200
HIVNL4.3R	reverse	GAGTCCTGCGTCGAGAGAGC	HIV-1 NL4-3	58	200
HIVNL4.3pr	probe	5’-FAM-CAGTGGCGCCCGAACAGGGA-TAMRA-3’	HIV-1 NL4-3	58	200
RnasePf	forward	AGATTTGGACCTGCGAGCG	RPP30	58	200
RnasePR	reverse	GAGCGGCTGTCTCCACAAGT	RPP30	58	200
RnaseP probe	probe	/5'HEX/TTCTGACCTGAAGGCTCTGCGCG/3'IABkFQ/	RPP30	58	200

Touchdown ddPCR

The comparison of different touchdown procedures revealed that the most optimal procedure consists of 30 PCR cycles at stringent conditions, allowing specific PCR amplification. Subsequently, 9 PCR cycles at more relaxed conditions were adequate to accumulate sufficient fluorescent signal to enable maximal discrimination of positive versus negative droplets.

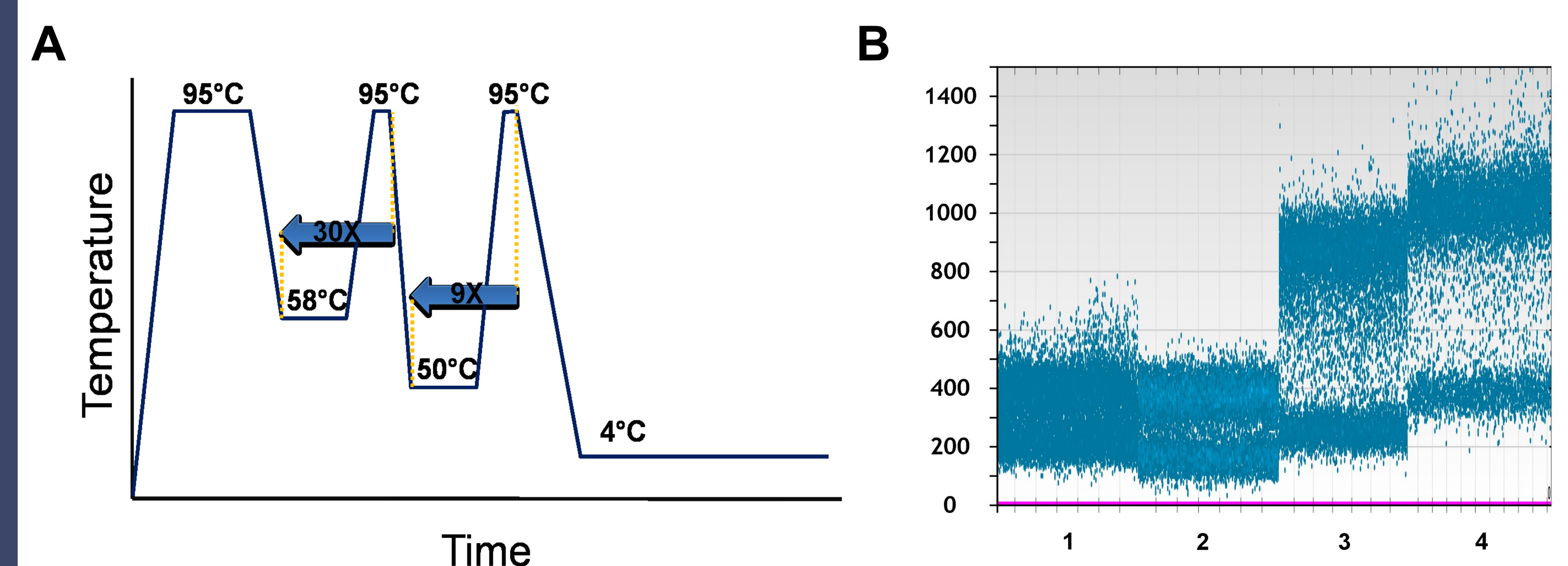


Figure 3: A) Schematic representation of the two stage touchdown procedure. The first 30 cycle are performed at stringent annealing temperature (Ta), the second round of 9 cycles at lower temperature. **B)** dot plot outputs of four different touchdown procedures: **1:** 30X 58°C & 6X 50°C; **2:** 33X 58°C & 6X 50°C, **3:** 24x 58°C & 15x 50°C, **4:** 30x 58°C & 9x 50°C. The distinction of positive versus negative droplets was optimal at condition 4.

Validation of the touchdown ddPCR

To validate the quantitative accuracy of the ddPCR, a two-fold standard dilution series of *in vitro* infected cells with HIV was prepared, with infection levels similar to observed *in vivo* infection levels.

Subsequently, HIV DNA was quantified in peripheral blood mononuclear cells (PBMCs) from HIV-infected patients to provide proof of concept that this method works on patient derived DNA . An internal reference gene (RPP30; table 1) was used to enable normalization of sample inputs.

PBMCs from 16 patients were procured. These consisted of 8 therapy naïve viremic or elite controllers and 8 samples from patients on therapy.

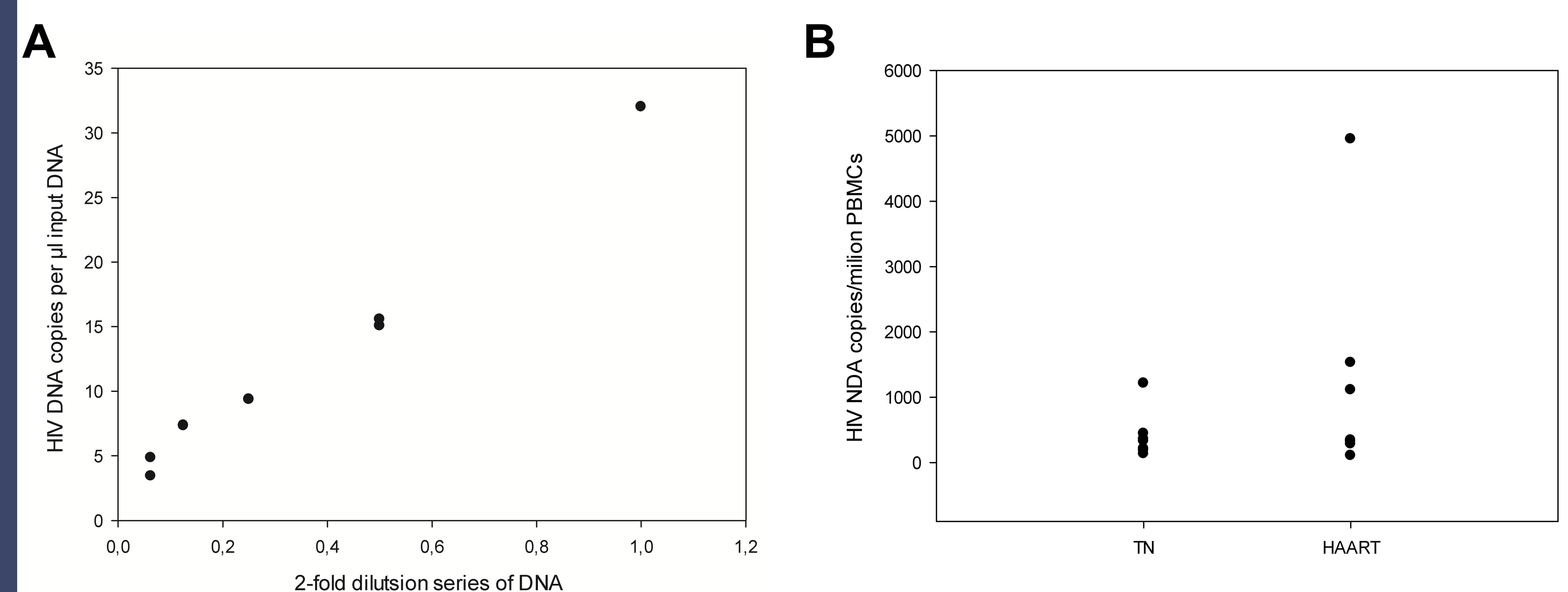


Figure 2: A) Quantification of a 2-fold dilution series in duplicate ddPCR reactions of DNA from infected cells, revealing quantitative accuracy of ddPCR measurements **B)** HIV DNA copies per million PBMCs in the 16 patients. No significant difference was observed between therapy naive viremic and elite controllers (TN), and between patients on highly active antiretroviral therapy (HAART).

DISCUSSION:

New diagnostic tools for HIV disease monitoring in virologically well suppressed patients are required in order to guide the development and use of new therapeutic strategies. Digital PCR is proposed as a promising technology for HIV DNA and RNA quantification as it provides a high quantitative accuracy at low levels of target nucleic acids.

The present work indicates that the high flexibility of assay design is an additional asset for the use of ddPCR. This will allow the development of assays specific to a larger variety of *in vivo* HIV variants, allowing a better assessment between patients infected with dissimilar HIV variants.

CONCLUSIONS:

- Digital PCR forms a promising technology for monitoring HIV DNA in patient samples.
- The digital droplet PCR technology allows a high flexibility in assay design without losing quantitative accuracy and specificity.

ACKNOWLEDGEMENTS: This study was supported by the American foundation for AIDS Rezsearch (amfAR)

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