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Triplex digital PCR assays for the quantification of intact proviral HIV-1 DNA

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ABSTRACT

The development of an HIV-1 cure is hampered by the existence of a persistent (latent) reservoir that contains a small proportion of replication-competent intact proviruses which refuels viral replication upon treatment discontinuation. Therefore, an accurate evaluation and quantification of these (intact) proviruses is essential to determine the efficacy of HIV-1 cure strategies which aim to eliminate this reservoir. Here, we present two triplex digital PCR assays which resulted from a combination of two existing methods, the IPDA (a 2-colour digital PCR based method) and Q4PCR assays (4 colour qPCR method), and tested the functionality on a three-colour digital PCR platform. In the present paper, we provide a step-by-step experimental protocol for these triplex digital PCR assays and validate their performance on a latently infected Jurkat cell-line model and HIV-1 patient samples. Our data demonstrates the potential and flexibility of increasing the number of subgenomic regions of HIV-1 within the IPDA to acquire sensitive detection of the HIV-1 reservoir while benefitting from the advantages of a dPCR setup.

1. Introduction

Since the introduction of (combination) antiretroviral therapy (ART), HIV-1 infection has turned from a deadly into a (manageable) chronic disease [1]. However, while ART is effective in suppressing viral replication in individuals, it does not offer a cure for HIV-1 infection. This is due to the existence of a persistent (latent) reservoir that contains a small proportion of replication-competent intact proviruses (1–5%) which refuels viral replication upon ART cessation [2–4]. Therefore, the efficacity of HIV-1 cure strategies aiming to eliminate this reservoir is dependent on an accurate evaluation of these intact proviruses.

Over the past years, several PCR-based methods coupled with Next Generation Sequencing (NGS) have emerged to detect intact proviruses [4–7]. Although these assays exert high sensitivity and can provide full-length HIV-1 sequences, they are currently not cost-efficient and often rely on manual limiting dilutions with lengthy labour-intensive protocols (reviewed in [8]). Recently, a duplex droplet digital PCR (dPCR) based assay has been described to quantify intact proviruses from HIV-1 patients, the intact proviral DNA assay (IPDA) [9]. This dPCR-assay quantifies two regions in the HIV-1 genome (the packaging signal (PSI) and the envelope (ENV)) that often contain defects such as deletions or hypermutations that interfere with PCR amplification so that detection is only possible when these regions are intact [9]. Hereby, this assay gives a better estimation of the number of intact proviruses in comparison to other assays targeting only one region. Several clinical studies have already shown the implementation of the IPDA to estimate the intact reservoir in patients. However, sequence variations in either one of the assays remains a problem for accurate detection of the intact provirus [10–15].

A second PCR-based approach using multiple subgenomic HIV-1 assays to assess if intact sequences are present in patient samples is the Q4PCR [16]. This workflow introduces a quadruplex qPCR in their

Abbreviations: ART, Antiretroviral therapy; dPCR, Digital PCR; DSI, DNA shearing index; ENV, *Envelope* protein sequence; FCS, Fetal calf serum; GAG, *Gag* protein sequence; gDNA, Genomic DNA; IPDA, Intact proviral DNA assay; NTC, No-template control; NGS, Next generation sequencing; PBMC, Peripheral blood mononuclear cells; POL, *Pol* protein sequence; PSI, Packaging signal; RPMI, Roswell Park Memorial Institute.

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(full-length) HIV-1 sequencing protocol to assess HIV-1 genome intactness before performing near-full length sequencing. However, the quadruplex qPCR is not optimized to use as a stand-alone absolute quantification method and the Q4PCR workflow remains labour-intensive due to the requirement of limiting dilution, near-full length HIV-1 PCR and subsequent sequencing.

The development of innovative techniques and platforms to improve the field of dPCR is rapidly evolving, especially the aspect of higher dimensional/multiplex dPCR capabilities. In this context, the Naica digital PCR system (Stilla) is capable of detecting three different fluorophores and simplifies the PCR setup to detect multiple targets [17]. Here, we describe the integration of the previously described IPDA and Q4PCR method on this dPCR platform, which increases the number of subgenomic regions of HIV-1 over the IPDA to acquire sensitive detection of the reservoir while benefitting from the advantages of a dPCR setup [9,16].

2. Materials and methods

2.1. Required reagents and equipment

- Primers (Integrated DNA Technologies, Inc. (IDT), USA)
- Probes (Integrated DNA Technologies, Inc, (IDT), USA)
- PerfeCta Multiplex qPCR ToughMix (5x) (Quanta Biosciences, USA, cat#: 95147–250)
- Nuclease-free water (Sigma Aldrich, Merck, Germany, cat# W4502-1L)
- Fluorescein (1 μM) (VWR International Bv, Belgium, cat#: 0681–100 g)
- Naica® Geode (Stilla technologies, France)
- Ficoll 400 (Merck, Germany, cat#: F4375)
- Prism3 instrument (Stilla technologies, France)
- Sapphire chips (Stilla technologies, France)
- Crystal Miner software (available on https://www.stillatechnologies.com/software/)
- AF advanced technology cleaning Screen-Clene (AF International, United Kindom, cat#: SCS250)
- Roswell Park Memorial Institute (RPMI) 1640 medium with GlutaMax (Gibco, ThermoFisher Scientific, USA, cat#: 61870036)
- Fetal calf serum (FCS) (Gibco, ThermoFisher Scientific, USA, cat#: A4766801)
- Penicillin-Streptomycin (10,000 U/mL) (Gibco, ThermoFisher Scientific, USA, cat#: 15140163)
- 1x IDTE (1X TE Solution) (Integrated DNA Technologies, Inc. (IDT), USA, cat#: 11-05-01-05)
- 10 μL pipet tips
- 100 μL pipet tips
- 200 μL pipet tips
- 1000 μL pipet tips
- 1,5 mL Eppendorf tubes (Eppendorf Belgium N.V., Belgium, cat#: 0030120086)
- 0,5 mL Eppendorf tubes (Eppendorf Belgium N.V., Belgium, cat#: 0030121023)
- DNeasy Blood & Tissue Kits (Qiagen, Germany, cat#: 69504)
- Qubit® 2.0 Fluorometer (ThermoFisher Scientific, USA, cat#: Q33327)
- Qubit® DNA BR Assay Kit (ThermoFisher Scientific, USA, cat#: Q33265)

2.2. Procedure

2.2.1. Sample preparation

As a positive control sample, the latently infected clonal Jurkat cell line 8.4 (J-Lat 8.4), which harbours one copy of the HIV-1 genome per cell, was obtained from the NIH Reagents Program and cultured in RPMI medium (10% FCS and 10 units/mL Penicillin-Streptomycin) at 37 °C and 5% CO_2 incubator [18]. HIV-1 patient sample collection involved PBMC isolation from whole blood by Ficoll 400 density gradient centrifugation and samples were stored as dry-frozen cell pellets (-80 °C). All patient samples were isolated after a signed informed consent and the approval of the Ghent University ethical committee (B670201525241) [19]. Patient samples from this study were selected based on HIV subtype B, reservoir size (determined previously) and availability of gDNA material. All patient samples, at the time of sampling, had an undetectable plasma viral load.

Genomic DNA (gDNA) extraction was performed using the DNeasy Blood & Tissue Kit, according to the manufacturer's instructions, and isolated from 1×10^6 J-Lat 8.4 cell pellets and 1×10^7 PBMC pellets from HIV-1 patients. DNA was eluted in 75 μL of nuclease-free water and the concentration measured on the Qubit® 2.0 Fluorometer using the Qubit® DNA BR Assay Kit following manufacturer instruction.

2.2.2. HIV-1 triplex and RPP30 duplex assay setup: Primer and probes

Primers and probe sequences for the HIV-1 subgenomic assays were obtained from Bruner *et al.* (2019) and Gaebler *et al.* (2019) (Table 1, Fig. 1) [9,16]. To combine these assays in a three colour dPCR system, the GAG and POL probes from Gaebler *et al* (2019) were modified to contain a Cy5 fluorophore while maintaining the IPDA assays with PSI-FAM and ENV-HEX [16]. The dark competition probe for APOBEC3G

Table 1
Primer and probe sequences used in the triplex HIV-1 DNA assays.

Primer name	Orientation	Sequence (5′ – 3′)	Reference
Primer PSI F	Forward	CAGGACTCGGCTTGCTGAAG	[9]
Primer PSI R	Reverse	GCACCCATCTCTCTCCTTCTAGC	[9]
Primer ENV F	Forward	AGTGGTGCAGAGAGAAAAAAGAGC	[9]
Primer ENV R	Reverse	GTCTGGCCTGTACCGTCGC	[9]
Primer GAG F	Forward	ATGTTTTCAGCATTATCAGAAGGA	[16]
Primer GAG R	Reverse	TGCTTGATGTCCCCCCAT	[16]
Primer POL F	Forward	GCACTTTAAATTTTCCCATTAGTCCTA	[16]
Primer POL R	Reverse	CAAATTTCTACTAATGCTTTTATTTTTTC	[16]
Probe PSI	Probe	/56-FAM/ TTTTGGCGTACTCACCAG/ MGBEc	[9]
Probe ENV	Probe	/HEX/CCTTGGGTTCTTGGGA/MGBEc	[9]
Probe GAG	Probe	/Cy5/CCACCCCAC/TAO/ AAGATTTAAACACCATGCTAA/IAbRQSp/ *	[16]
Probe POL	Probe	/Cy5/AAGCCAGGA/TAO/ATGGATGGCC/ IAbRQSp/ *	[16]
RPP30 F1	Forward	AGATTTGGACCTGCGAGCG	[20]
RPP30 R1	Reverse	GAGCGGCTGTCTCCACAAGT	[20]
RPP30 F2	Forward	GCTAACCTTTGATAGGCTCAGT	This work
RPP30 R2	Reverse	CAAATGGTAAGAAGGGCAGAAAC	This work
RPP30 Probe 1	Probe	/HEX/TTCTGACCT/ZEN/ GAAGGCTCTGCGCG/AbKFQ	[20]
RPP30 Probe 2	Probe	/56-FAM/AGCTAAGTG/ZEN/ TTGATCCATCTCTTTCTAGCT/AbKFQ	This work

*Alteration of the fluorophore of the original assay.

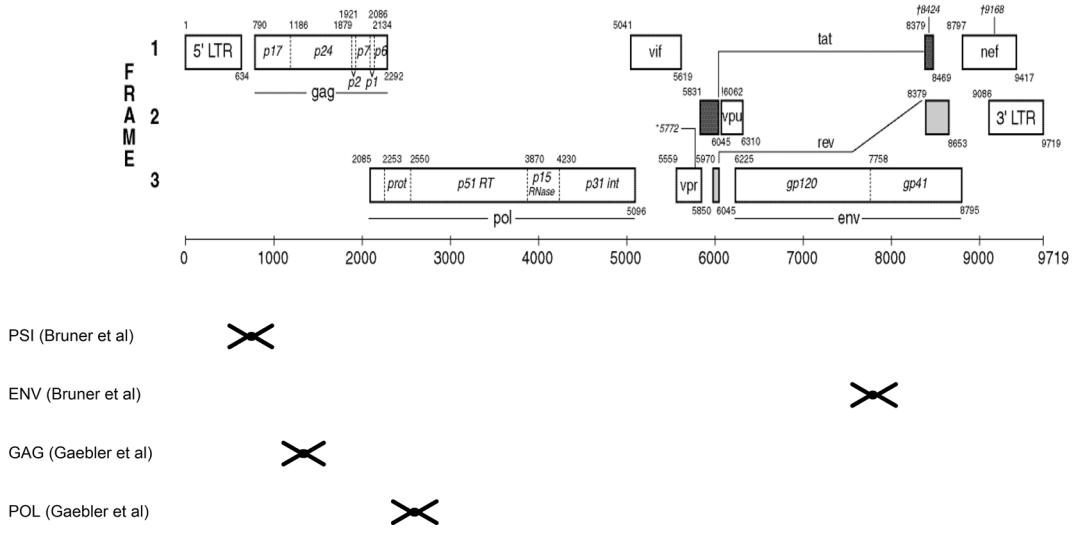

Fig. 1. Schematic overview of the assays on the HIV-1 genome. The assays used in this study are plotted at their respective locations on the HIV-1 genome. The location of the forward and reverse primers are depicted as arrows and the probe as a dot. In brackets next to the assays name the corresponding paper of origin, e.g. Bruner *et al.* (2019) or Gaebler *et al.* (2019) [9,16]. Abbreviations: PSI: Packaging signal, ENV: *envelope* protein sequence, GAG: *Gag* protein sequence, POL: *Pol* protein sequence.

hypermutations in the ENV region, as described by Bruner *et al.* was omitted from this setup [9]. Next to the HIV-1 triplex assays, the duplex RPP30 host genomic DNA assay as described by Bruner *et al.* (2019) was implemented (pair 1 from [20] and pair 2 own designs) to estimate and correct for DNA shearing during the technical procedures (i.e. DNA isolation) [9]. The reasoning for this is that shearing could account for the dropout of one of the HIV-1 DNA assays due to technical reasons rather than defects in the HIV-1 genome. All oligos were dissolved in 1x TE buffer to generate a stock at a final concentration of 100 μM. The primer stock was diluted 10-fold in nuclease-free water to obtain a 10 μM working solution.

2.2.3. Triplex HIV-1 DNA assays

For the triplex assays, an input was used of 50 ng for J-Lat 8.4 gDNA and 700 ng gDNA for patient samples. For the J-Lat spike-in experiment 36 ng or 3.6 ng of J-Lat 8.4 gDNA was spiked into a background of HIV-1 free SupT1 gDNA to a total of 700 ng input per reaction. All samples were run in duplicates. Additional notes and considerations for performing the dPCR are given in Section 2.2.6.

1. Prepare for each sample the following reaction mix for a 3-colour probe assay (Table 2) (for assays with fewer probes substitute the primer and probe amounts for sterile nuclease-free water).
2. Vortex the reaction mixes for 2 s and spin down, using a tabletop spinner.
3. For each sample distribute 24 μL into a 0,5 mL DNase free microcentrifuge tube.
4. Add 1 μL of the DNA sample to the mix. When low concentrations of DNA are observed before dPCR, water can be substituted for the DNA sample. As a no-template control (NTC) nuclease-free water was added instead of gDNA.
5. Vortex the sample and reaction mix after addition of the sample and spin down in a tabletop spinner.
6. Take a Sapphire chip and place this on a tissue before pipetting the samples. This step is not necessary and chips can be kept in the plastic tray they are provided in, however, for ease of use taking them out might be beneficial. Carefully handling the Sapphire chips is required to not damage the membrane under the chip, which will lead to interference during the readout.
7. Remove the white caps from the Sapphire chips.
8. Carefully pipet the reaction volume (25 μL) into each well of the Sapphire chips.
9. Seal the wells with a provided sealing cap and place the chip in the plastic tray.
10. Turn on the Naica® Geode.
11. Clean the underside of the Sapphire chips with Screen-Clean to get rid of dust particles.
12. Carefully place the Sapphire chips in de Naica® Geode.
13. Run the following PCR program (Table 3). The program takes about 2 h and 15 min to complete.
14. Switch on both the computer and the Naica® Prism3 reader.
15. Start the Crystal Miner software and copy the chip number into the program.

Table 2
Reaction mixture for triplex dPCR.

Material	Final concentration	Volume (μL)
PerfeCta Multiplex qPCR ToughMix (5X)	1 X	5
Fluorescein (1 μM)	100 nM	2,5
Primer ENV F ϕ	800 nM	2
Primer ENV R ϕ	800 nM	2
Primer PSI R ϕ	800 nM	2
Primer PSI F ϕ	800 nM	2
Primer GAG or POL F * ϕ	800 nM	2
Primer GAG or POL R * ϕ	800 nM	2
Probe ENV ϕ	400 nM	1
Probe PSI ϕ	400 nM	1
Probe GAG or POL * ϕ	400 nM	1
Nuclease-free water	–	1,5
Final volume		24

* = For inclusion of either the GAG or POL assay, use their respective primers and probes.
ϕ = Primers and probes are interchangeable with RPP30 primer and probes. For duplex assays, leave out one of the primer/probe pair and replace indicated volumes with nuclease-free water.

16. Carefully remove the chip from the PCR machine, and make sure the bottom is dust-free, by wiping off the underside of the chip using the tissue delivered with the chips.
17. Place the chip into the reader and close the Naica® Prism3 reader via the software.
18. Adjust the sample annotation e.g., sample names into the Crystal Miner software.
19. Adjust the laser exposure time following the designed assay, samples can be re-run with adjusted settings. Exposure times used for the triplex assays: FAM; 65 ms, HEX; 250 ms and Cy5 30 ms. Exposure times used for the RPP30 assays: FAM; 80 ms, HEX; 100 ms, Cy5; 30 ms.
20. Start the read-out. Read-out takes around 5 min to perform per loaded strip.
21. Perform data analysis in the Crystal Miner software.

2.2.4. Data analysis in the crystal miner software

Analysis of the data was performed in the Crystal Miner software and includes spillover compensation, thresholding and quantification.

2.2.4.1. Spillover compensation. Spillover compensation was performed for each respective assay using the build-in spillover compensation tool within the Crystal Miner software. To calculate the spillover matrix of both the triplex assays, we used the fluorescence intensity values of each assay included in the triplex but run as a singleplex control. Using these singleplex data the Crystal Miner software can compute a spillover compensation matrix as followed:

1. In the Crystal Miner software, navigate to the Setup tab.
2. In the Setup tab, select Compensate Spillover.
3. Generate the spillover compensation by pressing the compute button in the lower right panel.
4. Select the samples containing the singleplex assays and check the box for the respective fluorophore. A negative population can be determined by checking the NNN-box in a negative control but is not required.
5. Set the threshold for each respective fluorophore under the positive cloud and press compute.
6. The plots in the Setup tab, Compensate Spillover, have now be adjusted based on the set thresholds. Additional adjustments can be made by changing the numbers in the table in the lower right panel.
7. Press Apply to safe and apply the spillover compensation for all the samples. Safed spillover compensations can be applied to a certain assay after calibration.

Additional information on how the calculations for the spill-over compensation is extensively explained in [17].

2.2.4.2. Thresholding. Positive and negative partitions were discriminated by manual thresholding, after spillover compensation. The setting of the thresholds was performed by first setting the thresholds on negative controls in the 2D plots in Crystal Miner. Next, the thresholds were applied to the individual samples and adjusted if necessary to account for baseline shifts in negative and positive populations. The implementation of data-driven methods for threshold determination is preferred but are currently not compatible with the data format from the Stilla platform.

Table 3
PCR program in the Naica® Geode.

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Annealing	60	30 sec	

2.2.4.3. Quantification. The Crystal Miner allows for the identification of different populations of positive partitions. We used this to quantify the double and triple positive partitions in the proposed assays. Data was extracted from the software via the export function provided by the software. First, the copies/μL found quantified by the Crystal Miner software were converted to copies per reaction (25 μL) to determine the total quantity of copies of the input. Second, samples were corrected based on the DSI and total cells analysed, calculated from the RPP30 duplex assay (more details provided in Section 2.2.5). Finally, the data was visualized in R version: 4.0.2 using the following packages: tidyverse, ggthemes, ggpubr, scales, and igraph.

2.2.5. Duplex RPP30 host genomic DNA assay: DNA shearing compensation

2.2.5.1. Digital PCR procedure. The digital PCR for the RPP30 duplex assay was carried out in duplicate as described in Section2.2.3. but with 7 ng DNA input. Primers and probes for the triplex assay in the reaction mix (Table 2) were substituted for RPP30 primers and probes (Table 1) and additional nuclease-free water was added to get to a total volume of 24 μL.

2.2.5.2. Data analysis and DSI correction. Data analysis in Crystal Miner software, involved spillover compensation and thresholding as described in 2.2.4.Next, the compensated data was extracted from the Crystal Miner software. For each sample, the DNA shearing index (DSI) was calculated, as developed by Bruner *et al.* (2019), and beholds the ratio of the number of double-positive partitions and the average of the single positive partitions of a duplex digital PCR reaction of the RPP30 reference gene [9]. The distance between the two amplicons within the RPP30 duplex is the same as the distance between the PSI and ENV amplicons, allowing for the calculation of DNA shearing during the sample preparation. In this first correction, the concentration of the IPDA, triplex and duplex RPP30 assay is divided by 1 minus the DSI value in order to compensate for shearing. Based on the double-positive partitions in the RPP30 duplex, the second correction was made. Herein, the number of cells was calculated by dividing the double-positive copies with the number of gene copies of RPP30 per cell (2 copies for PBMCs and 4 copies for J-lats). With this, the total number of cells used in the input was calculated and subsequently used to calculate the copies of the IPDA and triplex assays in copies/million (patient samples) or copies/cell (J-Lat samples). During the study we found a DSI of on average of 0,29 for patient samples and a DSI of 0,34 for J-Lat 8.4, in line with DSI reported in Bruner *et al.* (2019) [9].

2.2.6. Additional notes and features

- For the Qiagen DNeasy Blood & Tissue kit, it is important to note that the AL and AW1 buffers are not compatible with bleach and should not be discarded as such. Waste containing the buffers should be discarded in the not hazardous waste or via sink disposal.
- When pipetting the sample into the Sapphire chips, make sure the chip is not in contact with a rough surface. Failing to do so could damage the chip and lead to incorrect or no read-out by the Prism3 instrument.
- Before starting the PCR protocol make sure that the vacuum pump is switched on, allowing for the correct pressure inside the machine and generation of droplets.
- Generation of droplets occurs during the first step in the Naica® Geode. To make sure droplets are generated correctly, the Naica® Geode must be grounded before placing the chips into the machine to prevent static electricity from interfering with droplet generation. Grounding the machine can be done by simply touching the machine with bare skin.

- Exposure times of the lasers can be adjusted before reading out the samples. Exposure times may be different for different assays and should be evaluated.
- Chips can be re-run on the Prism3 reader, for example, to get rid of potential dust on the chip or adjusting the read-out parameters, e.g. exposure time. It is recommended not to exceed to do more than 5 read-outs on a sample, this might bleach the probe fluorescence.
- If downstream applications are required on the sample, e.g. for sequencing, droplets can be reobtained from the Sapphire Crystal. The protocol to do so is provided on the Naica® website: https://www.stillatechnologies.com/droplet-recovery-with-crystal-digital-tm-pcr/

3. Results and discussion

3.1. Performance of triplex assays to quantify HIV-1 DNA in J-Lat 8.4

For the initial validation of the triplex assays, we performed singleplex reactions of each assay, the original two-colour IPDA assay and the two triplex assays on 50 ng of J-Lat 8.4 gDNA in two separate experiments (Fig. 2, Supplementary Fig. 1). Performance of the assays was visually inspected and all assays resulted in separated and distinguishable clouds of negative and positive partitions (Supplementary Fig. 1). Singleplex reactions were only used to calculate a spill-over matrix for compensation purposes and quantification results of individual signals are within the context of the triplex assay reactions.

3.1.1. Quantification

All individual signals within the triplex assays gave similar quantification results (difference between triplex assays: 1,19% total PSI, 1,69% total ENV and 3,3% between total GAG and POL) with the total PSI assay retaining the highest signal (Fig. 2 A). Analysis of the IPDA positive and triplex positive signals show that most of the found sequences are intact based on a two or three region detection (94,5 ± 3,5% IPDA, 91,6 ± 7,0% triplex GAG, 93,4 ± 2,4% triplex POL) (Fig. 2 B). Correction of DNA shearing using the DSI led to a very good estimation of the expected HIV-1 copies present in J-Lat cells (expected to contain 1 copy per cell) when applied to the IPDA and triplex assays (Fig. 2 A). Indicating, that DNA shearing is indeed occurring in the sample preparation and should be accounted for during quantification.

Furthermore, we observed a small variation (2,5%) between the GAG and POL triplex in terms of triple-positive copies, indicating that both assays make a similar estimation of intact sequences in J-Lat DNA (Fig. 2 B). It has to be noted that for the J-Lat cells we expected a fairly large proportion of the sequences to be intact, and thus no substantial differences between the triplex assays and the IPDA were expected.

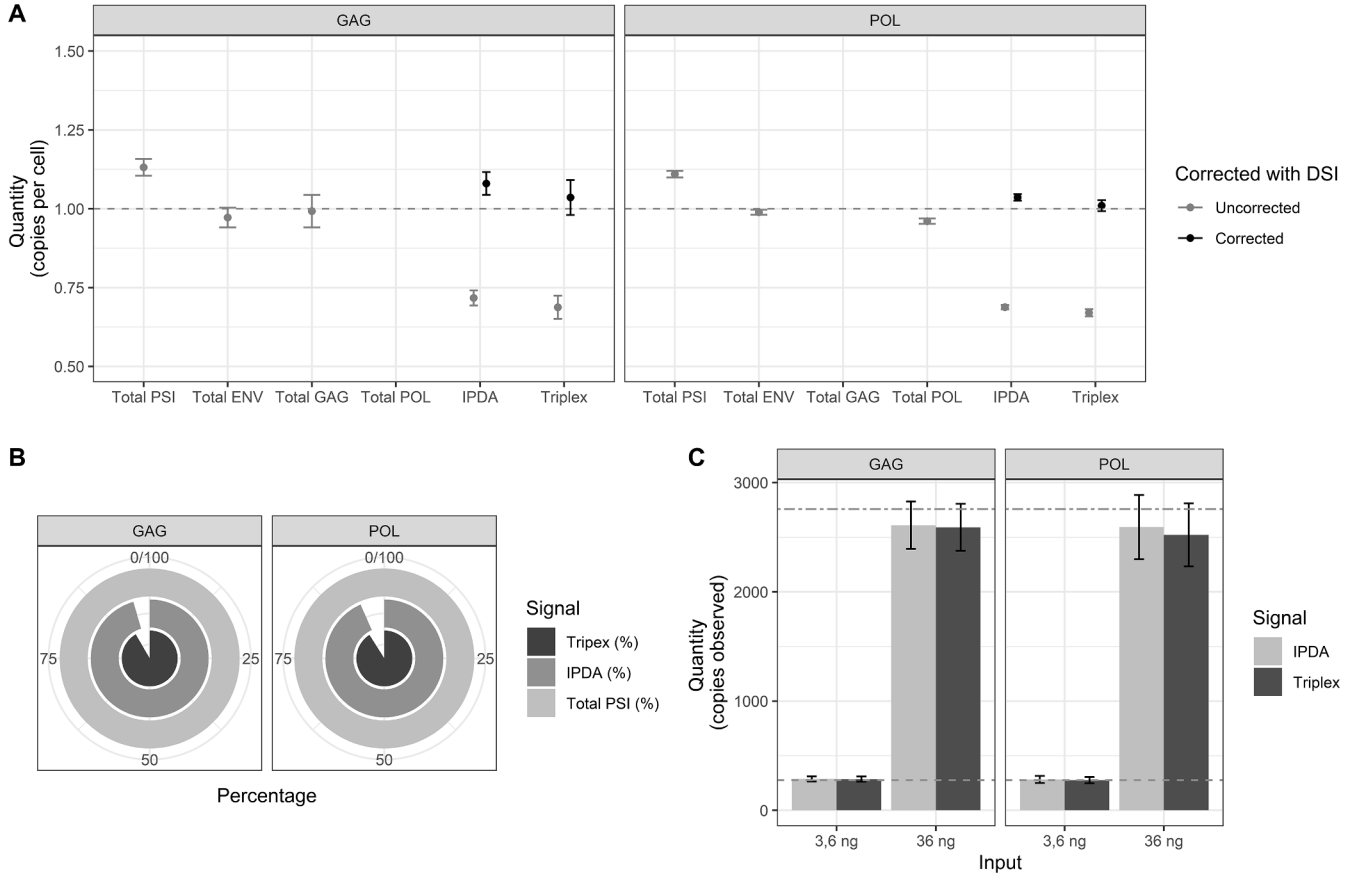

Fig. 2. Assay performance on J-Lat 8.4 genomic DNA. A) Quantified copies per cell in J-Lat 8.4 cells. Copies per cell were quantified for the signals of the individual assays (total PSI, ENV, GAG/POL, IPDA and triplex) within the 2 triplex assays. The copies per cell for the IPDA and triplexes are shown as both corrected with the DSI and without, showing the effect of correcting for shearing. J-Lat samples were performed in duplicate using 50 ng input. Error bars indicate the error between replicates. The dotted line represents the expected copies/cell in J-Lat 8.4 (1 copy/cell expected). B) Percentage of observed IPDA and Triplex copies as a proportion of the copies quantified for PSI in J-Lat samples within the triplex assays. The results are shown for both the GAG and POL triplex assays. C) Observed HIV-1 copies when spiked into negative background DNA. 36 and 3,6 ng of J-Lat 8.4 DNA was spiked into a total of 700 ng of SupT1 gDNA and run twice in duplicates. Copies were quantified for the IDPA and triple-positive signal (triplex) in both triplex assays after DSI correction. Error bars indicate the error between replicates. Expected copies, calculated from the input, are shown in the dashed line for the 3,6 ng input and the double dashed line for the 36 ng input. Abbreviations: PSI: Packaging signal, ENV: *envelope* protein sequence, GAG: *Gag* protein sequence, POL: *Pol* protein sequence, IPDA: intact proviral DNA assay, DSI: DNA shearing index.

3.1.2. Precision and repeatability

In terms of precision and repeatability, both triplex assays exert an acceptable coefficient of variation (CV) lower than 5% (Table 4). The triplex POL assay shows better performance over the triplex GAG assay and is comparable to the two-colour IPDA. The triplex GAG assay shows a higher CV but is well within an acceptable range lower than 5%. These CV values are in line with the CV values found for the IPDA and indicate that the triplexes performed similarly in terms of precision and repeatability [9].

Next, we looked to determine the precision and repeatability in a more complex sample matrix, for this, we spiked 36 ng or 3,6ng of J-Lat 8.4 gDNA into a background of SupT1 gDNA to a total of 700 ng input and performed the triplex assays (Fig. 2 C). Spike-in experiments were carried out twice in duplicate for each concentration. In terms of quantification, we observed minimal differences between the expected and observed copies in the IPDA and the triple-positive signal of both triplex assays. The maximum difference found for both the IPDA and triple-positive signals respectively were 5,4 and 6,0% in the GAG triplex and 6,0 and 8,6% in the POL triplex (Fig. 2 C). In terms of precision, the GAG triplex showed a slightly lower coefficient of variation values compared to the POL triplex for both the IPDA and triple positive signals (Table 5). The CV% is retained for both the IPDA and triple-positive signals after diluting the input 10 fold in both triplex assays.

3.2. Performance of triplex assays to quantify intact HIV-1 DNA in patient samples

To get insight into the functionality of both triplex assays in more complex samples, we applied the workflow to five PBMC samples from HIV-1 patients with an undetectable viral load and are under ART (Supplementary Table 1). Quantification results were DSI corrected and expressed as copies per million PBMCs (Fig. 3 A). For samples in which no double or triple positive partitions were found, no DSI correction was made.

3.2.1. Overall performance

Using the triplex assays on patient samples, we could detect HIV-1 in 5/5 patient samples with at least 2 of the 4 assays incorporated in the triplex assays. Whereas double and triple positive signals in the triplexes were only detected for 3/5 and 3/5 in the GAG triplex and 2/5 and 2/5 in the POL triplex (Fig. 3A). This indicates the flexibility and the potential of incorporating additional regions into the IPDA setup. The signal of triple-positive sequences (% from the maximum total HIV-1 copies sequences) found in patients was between 6,0 and 6,25% for the GAG triplex and between 8,0 and 8,3% for the POL triplex (Fig. 3 B).

Dropout was only observed for ENV signal in 1 patient sample and no dropout for the other assays. Indicating potential defects in that region but sequence variation that causes PCR failure due to mismatches in the primer/probe region has to be taken into account while interpreting these dropouts (see Section 4.1).

Similar to the Q4PCR we observed the same decline of signal in triple-positive partitions in comparison to double positives (Supplementary Table 2 and Supplementary Table 3) [16]. Next to this we also observed that the overall quantities of the IPDA signals were lower in comparison to other double-positive signals in terms of absolute frequencies.

Table 4
Coefficient of variation of the different signals within the triplex assays in J-Lat DNA.

	Coefficient of variation (%)				
	Total PSI	Total ENV	Total GAG/POL	IPDA	Triplex
GAG triplex	2,36	2,36	5,18	1,34	3,78
POL triplex	0,94	0,94	0,93	0,51	0,54

3.2.2. Patient-specific observations

In two patients, SLR_31 and SLR_40, we were able to quantify all the incorporated assays of the triplexes. Furthermore, quantification of the IPDA and triple positive partitions was possible in these patients (Fig. 3). The level of intact provirus compared to the maximum copies of HIV-1 found for these patients were between 6,0% and 10,8% in both triplexes (Fig. 3 B). Next to this, we observed the dropout of the ENV assay in SLR_26 and a very low signal (4 partitions in total) for the PSI assay, but not the GAG or POL. Indicating that there might be deletions or mutations in the PSI and ENV sequence, but that HIV-1 DNA is present in the sample. This shows that the addition of a third assay yields additional information which is especially beneficial in samples where the dropout of 2 assays is observed and could potentially be dismissed as negative based on the dropout of only 2 assays. Nevertheless, the dropout of an assay still indicates the mutation and/or deletion of the region. Finally, in SLR_29we observed the quantification of PSI, ENV and GAG and POL, but did not observe positive partitions for the IPDA and triple positives in either the GAG or POL triplex. Similar results were found for SLR_85 but positive partitions for the IPDA in the GAG triplex were observed. This could indicate that in these patients no or very little intact HIV-1 sequences were present or that the sequences have a high sequence variation and/or fragmentation. However, because no dropout of the single assays was observed further investigation is required for these particular patients to give a definitive conclusion about the intactness of the reservoir.

4. Considerations and concluding remarks

4.1. Sequence variation

The strength of a PCR-based assay lays within the primer and probe sequences used and that needs to be considered when analysing this type of intactness data. In a recent study by Kinloch *et al.* (2021), it has been shown that genetic variants in the HIV-1 genome might contribute to an underestimation of intact HIV-1 proviruses when using the IPDA assay in clinical studies due to the existence of primer and/or probe mismatches [10]. In addition, different HIV-1 subtypes will require specifically designed multiplex intact DNA assays, as these assays cannot be easily transferred between subtypes.

This sequence variation might also be a factor in our HIV-1 patient samples (2/5, SLR_26 and 29), as we also observed failure to detect any signal or fairly low quantities for PSI or ENV. Therefore, the addition of multiple HIV-1 regions such as the GAG and POL assays in these triplex approaches increase the information on the HIV-1 DNA reservoir. Indeed, in the study of Gaebler *et al.* (2020), it was shown that the addition of extra assays led to a better estimation of the reactivatable provirus population, indicating a more sensitive quantification of intact provirus [15].

4.2. Implementing the DNA shearing index

The DNA shearing index, described by Bruner *et al.* (2019), is an attempt to correct for DNA breaks or defects that arise during the technical procedure of the workflow which can induce an underestimation of the measurement of intact HIV-1 sequences [9]. When applying this DSI correction and interpreting the corrected quantification results, caution is advised, especially in the context of negative quantification results or high DSI values, where applying the correction can bias the end result. Therefore, we advise that the DSI should always be reported and described how this is applied by the users of these assays. For instance, we decided not to correct a 0-value result for DNA shearing and set a DSI maximum of 50% shearing as quality control. Furthermore, no DSI correction should be applied to samples in which the amount of positive partitions exceeds 30% of the total amount of partitions. Doing so would lead to an underestimation of the double and triple positive partitions, due to the increased possibility of partitions

Table 5
Coefficient of variation of the IPDA and triple-positive signal within the triplex assays in J-Lat DNA spiked in SupT1 DNA.

Signal	Input		IPDA			Triple-positive		
Assay	Input (ng)	Expected copies	Average (copies observed)	Sd	CV%	Average (copies observed)	Sd	CV%
GAG triplex	36	2758	2610	217,3	8,3	2592	215,0	8,3
	3,6	275	289	23,9	8,3	287	24,5	8,5
POL triplex	36	2758	2594	293,9	11,3	2522	289,0	11,5
	3,6	275	283	32,2	11,4	277	29,4	10,6

Abbreviations: Sd; standard deviation, CV%: coefficient of variation in percentages, IPDA: intact proviral DNA assay.

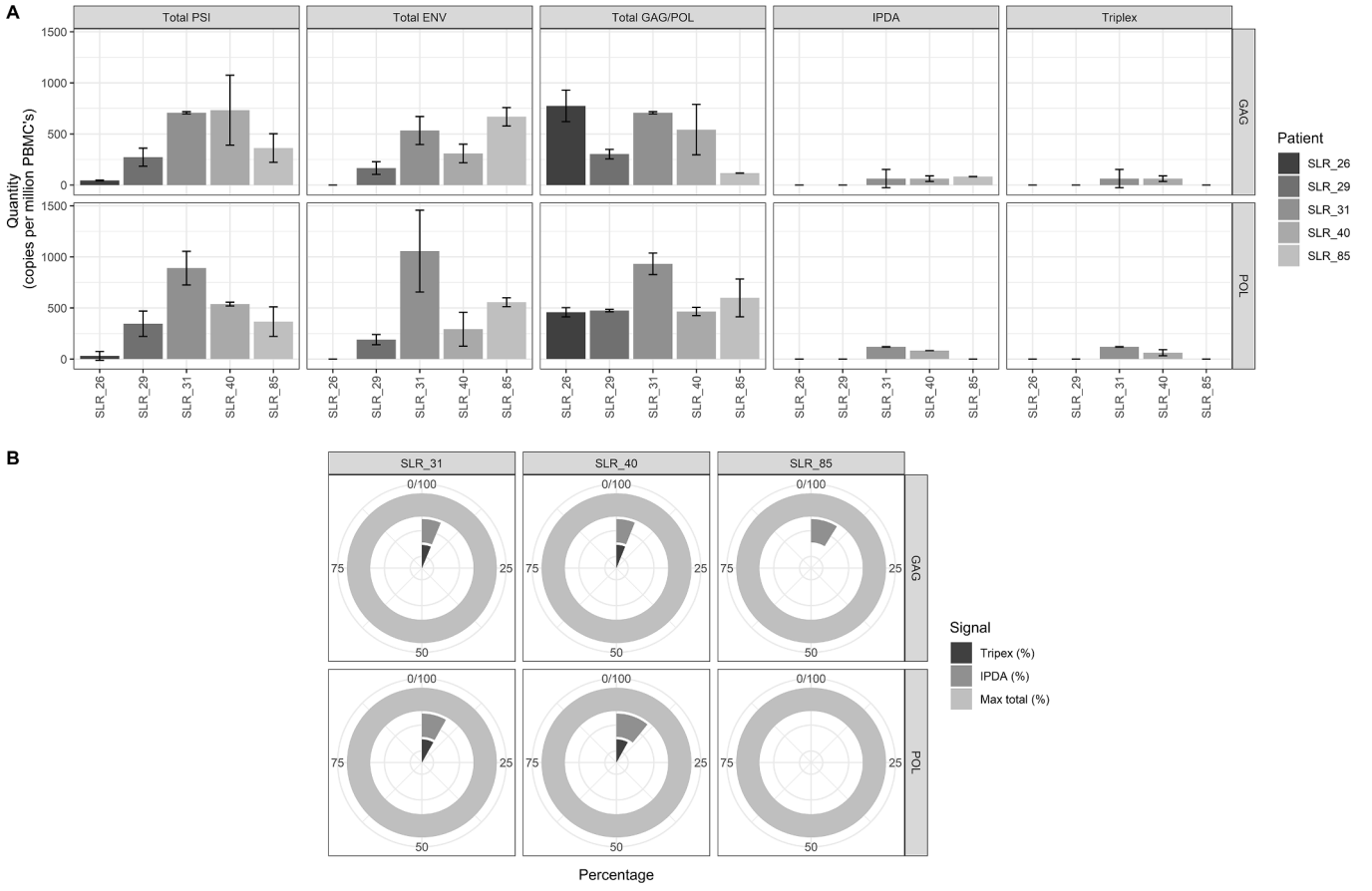

Fig. 3. Performance of the triplex IPDA assays on patient samples. A) Quantified copies per million PBMC's obtained from clinical samples. For all patients the quantities of two replicate are given for the signals within the triplex assays; total PSI, total ENV, total GAG/POL, IPDA and Triplex. The upper row shows the results in the GAG triplex whereas the lower row shows the results of the POL triplex. Collums represent the quantification of the different signals within the respective triplex assay, thus the quantified copies in each triplex assay. Samples are run in duplicate each duplicate containing 700 ng genomic DNA. The error bars indicate the spread between replicates. B) Percentage of IPDA and triple-positive copies in comparison to the maximum quantified HIV-1 copies in patient samples. For each patient, the results are shown for both the GAG and POL triplex. Patient samples were no partitions were observed for the IPDA and/or triplex assays are omitted. Abbreviations: PSI: Packaging signal, ENV: *envelope* protein sequence, GAG: *Gag* protein sequence, POL: *Pol* protein sequence, IPDA: intact proviral DNA assays.

containing multiple copies.

4.3. Low copy numbers and clinical implementation

Finally, as with other HIV-1 reservoir measurements or sequencing results, interpreting very low and/or no quantification results for HIV-1 intact sequences does not necessarily implicate an absence of intact HIV-1 sequences in the human body. For instance, in HIV-1 cure intervention studies where patients under ART might have very small intact latent reservoirs which can be missed in a limited sampling from the blood. Therefore, caution is required when data needs to be reported back to the clinic when it comes to this sort of assays. For patients in which a low reservoir is present, enrichment of $CD4^+$ cells could increase the signal in the triplex assays. Enrichment is especially advised in patients that have been under therapy for a long time. Next to this, as mentioned in Section 4.1., these intactness assays were designed to cover subtype B HIV-1 viruses, therefore negative results originating from different subtypes should also be further investigated and preferably analysed with subtype-specific versions of these intactness assays.

4.4. Conclusion

Assessment of the intactness of integrated provirus is crucial for the evaluation and development of potential HIV-1 cures. In this study, we (successfully) implemented triplex digital PCR assays to quantify HIV-1 DNA reservoirs from HIV-1 patient samples by integrating previously described IPDA and Q4PCR methods on a three-colour dPCR platform. This approach increases the number of subgenomic regions of HIV-1

over the IPDA to acquire sensitive detection of the reservoir while benefitting from the advantages of a dPCR setup. Notwithstanding the limited patient samples included in the study, we show the potential of multiplex quantification assays in the analysis of the HIV-1 reservoir, as these intactness assays can even be further scaled up in dPCR systems that enable 5 or 6 colour multiplexing.

CRediT authorship contribution statement

Willem van Snippenberg: Writing - original draft, Visualization, Investigation. **David Gleerup:** Investigation, Writing - review & editing. **Sofie Rutsaert:** Resources, Writing - review & editing. **Linos Vandekerckhove:** Supervision, Writing - review & editing. **Ward de Spiegelaere:** Methodology, Funding acquisition, Supervision. **Wim Trypsteen:** Conceptualization, Writing - review & editing, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2021.05.006.

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