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(54) **Title:** METHODS FOR SPECIFIC QUANTITATION OF THE INDUCIBLE HIV RESERVOIR

(57) **Abstract:** The present invention provides a method for determining whether a sample comprises inducible HIV-1, the method comprising performing a reverse transcription, loop-mediated isothermal amplification (RT-LAMP) reaction with said sample with a primer set specific for Tat/Rev multiply spliced (ms) HIV-1 RNA and determining whether the sample comprises an amplification product of the RT-LAMP reaction, wherein the binding sites for the F2 and F1 primer or for the B2c and B1c primer of said primer set span a region in said Tat/Rev ms HIV-1 RNA that overlaps with the splice site of the intron interrupting the Tat and Rev coding sequences.

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Title: Methods for specific quantitation of the inducible HIV reservoir

FIELD OF THE INVENTION

The invention is in the field of diagnostics. In particular, the invention pertains to methods for quantifying the persistent HIV-1 reservoir in individuals on suppressive antiretroviral therapy.

BACKGROUND OF THE INVENTION

Combination antiretroviral therapy (cART) has changed human immunodeficiency virus type-1 (HIV-1) infection from a lethal to a chronic illness. However, despite over 30 years of research, there is currently no safe, scalable, curative therapy in sight. Furthermore, HIV-1 treatment disparities persist, with approximately 70% of all individuals with HIV-1 residing in Sub-Saharan Africa, and only 78% with access to treatment. While standard clinical assays have shown that cART can effectively reduce circulating virus in the blood to undetectable levels, HIV-1 still persists in the form of an integrated provirus within a subset of immune cells, particularly resting memory $CD4^+$ T cells (the viral reservoir). If suppressive cART is interrupted, inducible replication-competent HIV-1 provirus (hereafter referred to as the inducible viral reservoir) can reactivate and exponentially replicate leading to detectable levels of viremia (viral rebound), within two weeks, in a majority of individuals. Lifelong therapy is thus necessary to maintain viral suppression, and interventions beyond cART are urgently needed to achieve an HIV-1 cure.

Toward this aim, several strategies that target the viral reservoir for elimination or control are being explored. To understand HIV-1 reservoir persistence, reliable biomarkers of viral reservoir size and dynamics are needed to evaluate the potential impact of interventions that aim to reduce the size of the latent viral reservoir. Furthermore, robust, precise, and clinically scalable assays capable of measuring the frequency of infected $CD4^+$ T cells containing inducible, replication-competent HIV-1 are

required. However, the low frequency of these cells, estimated at between 1 and 1000 per million $CD4^+$ T cells, makes quantification extremely challenging, highlighting the need for innovative solutions to identify, target and quantify the viral reservoir in the quest for an HIV-1 cure.

Numerous techniques have been proposed, developed, or used to measure the HIV-1 viral reservoir. PCR-based assays offer a quick and easy method to determine the frequency of cells containing total or integrated HIV-1 DNA. However, these assays considerably overestimate the size of the viral reservoir. By targeting only one highly conserved proviral region, defective HIV-1 genomes that harbor large deletions or are hypermutated in regions external to the assay amplicon are inclusively detected. Recent technological advances have addressed this issue by introducing assays that enable the detection of more than one conserved region of the HIV-1 genome. The Intact Proviral DNA Assay (IPDA), for example, targets the packaging signal (PSI) and env-RRE regions using ddPCR technology. Although it succeeds in excluding the majority of defective viruses, conventional IPDA targets a very small sub-genomic region (2% of the HIV-1 genome), which can lead to an overestimation of the viral reservoir. Adapted multiplex versions of IPDA have integrated additional assay targets for regions in HIV-1 Gag and Pol genes. A comparable technique to assess HIV-1 genome integrity, called Q4PCR, used a combination of a quadruplex qPCR and next-generation sequencing approach. While these assays have revealed novel insights into HIV-1 proviral landscape dynamics, some of the techniques are expensive, labor-intensive, and require sophisticated equipment, making them unsuitable for use in large-scale clinical trials, especially in resource-limited epidemiological settings where the HIV-1 pandemic is more prevalent. In addition, these assays exhibit high assay fail rates due to HIV-1 sequence polymorphisms within the primer-probe binding regions. Finally, proviral DNA sequence intactness is not a measure of viral inducibility, which greatly depends on

the site of viral integration in the host genome and therein the potential for viral transcription.

On the opposite end of the spectrum of reservoir quantitation methods, the culture-based, quantitative viral outgrowth assay (QVOA) is considered a gold standard technique to detect the outgrowth of replication-competent virus and therefore measures the functional viral reservoir. However, QVOA underestimates the size of the viral reservoir due to suboptimal induction and/or inadequate propagation of all replication-competent viruses *in vitro*. Moreover, QVOA requires a high input of $CD4^+$ T cells that necessitate large volume blood draws (>100 mL), is labor-intensive and time-consuming (14 to 21 days), limiting its use in routine, clinical settings.

Novel culture-based techniques have emerged as potential alternate assays to estimate the size of the inducible viral reservoir. These methods involve the activation of resting or total $CD4^+$ T cells from virally suppressed individuals, followed by the direct measurement of HIV-1 RNA from cell extracts (cell-associated RNA, ca-RNA) or cell culture supernatants (cell-free RNA, cf-RNA). However, it should be noted that assays relying on quantification of cf-RNA may not accurately assess replication-competency as it only reflects the capacity to generate and release viral RNA. Inducible ca-RNA assays, on the other hand, involve the potent activation of $CD4^+$ T cells by anti-CD3/CD28 antibodies, phorbol 12-myristate 13-acetate (PMA) and/or ionomycin, or latency reversing agents (LRAs), followed by an ultrasensitive PCR specific for a given viral transcript. Different RNA species including unspliced RNA (us RNA), multiply spliced RNA (msRNA), mature RNA transcripts with poly-A tails, TAR RNAs, and chimeric host-HIV-1 read-through transcripts can be quantified by RT-qPCR or RT-ddPCR methods. Tat/Rev msRNA in particular has been found to be a meaningful indicator of viral replication following latency reversal since Tat/Rev RNA transcripts are generated after splicing of full-length viral transcripts. Through the detection of Tat/Rev msRNA, the likelihood of measuring

proviruses with large internal deletions is greatly reduced. Inducible HIV-1 RNA assays can be conducted in a limiting dilution format, such as the Tat/Rev Limiting Dilution Assay (TILDA), or at single-cell level e.g., viral RNA detection by fluorescent in situ hybridization assays coupled to flow cytometry (FISH-flow). Single-cell approaches, such as FISH-flow, provide a deeper understanding of viral reservoir molecular characteristics and cell phenotypic heterogeneity but the sample processing conditions result in significant loss of sample thereby demanding high cell input (obtainable from >50 mL of blood). This important limitation makes FISH-flow assays less suitable for use in large scale cure intervention trials, and for this reason, viral RNA transcripts are preferably measured in bulk or in serial-diluted samples.

Quantification of HIV-1 RNA is typically performed using a single round or semi-nested quantitative reverse transcription real-time PCR (RT-qPCR). Semi-nested RT-qPCR is especially useful for assessing samples with low copies of the target RNA and offers a wider dynamic range compared to single-round qPCR. However, when HIV-1 RNA is quantified in limiting dilution format, e.g., 96 wells or 384 wells, the cost of reagents significantly increases. Moreover, the instrument-intensive amplification procedure, long turnaround time and high risk of cross-contamination when manually handling pre-amplified product in semi-nested RT-qPCR protocols limits assay application in large clinical trials, especially in resource-constrained settings.

There exists a great need for the development of highly sensitive methods for quantitation of cellular forms of HIV-1 RNA/DNA, in particular of cell-associated HIV-1 RNA in multiply spliced RNA (msRNA) form as the expression of msRNA species that encode Tat and Rev proteins may be linked to productive infection. Such methods should take the extremely low copy numbers of HIV-1 RNA/DNA in PBMC under antiretroviral therapy into account, in order to allow for systematic studies of the relationships between the cellular HIV-1 RNA/DNA levels and therapy outcome.

SUMMARY OF THE INVENTION

The present inventors have now found that reverse transcription, loop-mediated isothermal amplification (RT-LAMP) of Tat/Rev multiply spliced (ms) HIV-1 RNA is possible with very high sensitivity and specificity and that selection of LAMP primers to specific target regions of the template result in selective amplification of the multiply spliced target of interest over the unspliced RNA and DNA. The assay therefore allows for reverse transcription and LAMP in a single reaction performed directly on a sample comprising intact T-cells.

Some further advantages of the present invention as provided herein include the fact that the overall amplification process is fast, in that it takes less than 1 hour to even less than 30 min for completion of the reaction. The methods of the present invention preferably do not comprise the step of separate cDNA synthesis and amplification which greatly reduces the chance for the occurrence of cross contamination. The methods greatly reduce the hands-on time. The workflow of the amplification and detection process is less complicated than that of the prior art methods. The amplification and detection process is more tolerant to sample matrix inhibitors compared to conventional PCR. Moreover, there are less instrument constraints.

In a first aspect, the present invention provides a method for determining whether a sample comprises inducible HIV-1, the method comprising performing a reverse transcription, loop-mediated isothermal amplification (RT-LAMP) reaction with said sample with a primer set specific for Tat/Rev multiply spliced (ms) HIV-1 RNA and determining whether the sample comprises an amplification product of the RT-LAMP reaction. It is a feature of this aspect of the present invention that the binding sites for the F2 and F1 primer or for the B2c and B1c primer of the primer set span a region in said Tat/Rev ms HIV-1 RNA that overlaps with the splice site of the intron interrupting the Tat and Rev coding sequences.

In another aspect, the present invention provides a method of detecting an amplification product in a sample, the method comprising performing an RT-LAMP reaction with said sample with a primer set specific for Tat/Rev multiply spliced HIV-1 RNA and determining whether the sample comprises an amplification product of the amplification reaction. Again, it is a feature of this aspect of the present invention that the binding sites for the F2 and F1 primer or for the B2c and B1c primer of the primer set (or the F2 and F1 primer binding regions and the B1c B2c primer binding regions, respectively) span a region in said Tat/Rev ms HIV-1 RNA that overlaps with the splice site of the intron interrupting the Tat and Rev coding sequences.

In preferred embodiments of the above two method aspects of this invention, the primer binding sites for the F2 and B2c primers are located in the region defined by nucleotide positions 55 to 375 of the sequence of said Tat/Rev ms HIV-1 RNA.

In alternative or further preferred embodiments of the above two method aspects of this invention, the primer binding site for the F2 primer is located in the region defined by nucleotide positions 125 to 240 or preferably wherein the primer binding site for the B2c primer is located in the region defined by nucleotide position 190 to 305 of the sequence of said Tat/Rev ms HIV-1 RNA.

In still other alternative or further preferred embodiments of the above two method aspects of this invention, the distance between the F1 and F2 regions and/or between the B1c and B2c regions is at least 22 nucleotides.

In preferred methods of the present invention, said primer set comprises:

i) a forward inner primer (FIP) comprising at least two regions F2 and F1c, wherein F1c is linked to the 5'-side of F2, and wherein F2 is a region having a nucleotide sequence complementary to a region F2c in said Tat/Rev ms HIV-1 RNA sequence, and F1c is a region having substantially

the same nucleotide sequence as a region F1c located at the 5'-side of the region F2c in said Tat/Rev ms HIV-1 RNA sequence;

ii) a backward inner primer (BIP) comprising at least two regions B2 and B1c, wherein B1c is linked to the 5'-side of B2, and wherein B2 is a region having a nucleotide sequence complementary to a region B2c in a complementary chain synthesized from said Tat/Rev ms HIV-1 RNA sequence with the forward inner primer as the origin of synthesis, and B1c is a region having substantially the same nucleotide sequence as a region B1c located at the 5'-side of the region B2c in said complementary chain;

iii) a forward outer primer (F3) having a nucleotide sequence complementary to a region F3c located at the 3'-side of the region F2c in said Tat/Rev ms HIV-1 RNA sequence;

iv) a backward outer primer (B3) having a nucleotide sequence complementary to a region B3c located at the 3'-side of the region B2c in said complementary chain;

v) a forward loop primer (LF) having a nucleotide sequence complementary to a region LFc in said Tat/Rev ms HIV-1 RNA sequence located between the regions F1c and F2c;

vi) a backward loop primer (LB) having a nucleotide sequence complementary to a region LBc located between the regions B1c and B2c in said complementary chain.

In other preferred methods of the present invention, said sequence of said Tat/Rev ms HIV-1 RNA is the consensus sequence of *in silico* spliced Tat/Rev ms HIV-1 RNA sequences of a plurality of distinct HIV-1 isolates of a single HIV-1 subtype. In aspects of this invention, the consensus sequence is preferably an assembly of the nucleotides at each position within a multiple sequence alignment of a plurality of *in silico* spliced Tat/Rev ms HIV-1 RNA sequences wherein the nucleotide that appears common to a plurality of distinct HIV-1 isolates represents the corresponding nucleotide in that position of the consensus sequence.

In still other preferred methods of the present invention, the LF or LB loop primer comprises a cytosine (C) or guanine (G) residues at its 3′-end that represent a conserved nucleotide in said consensus nucleic acid sequence, and wherein the LF and/or LB loop primers comprise a thymine (T) residue at the second or third position from the 3′-end, preferably wherein the LF or LB loop primer sequence is used as the target-specific sequence of a self-quenching probe or molecular beacon. In a molecular beacon, the loop primer sequence forms the target-specific part of the molecule, which in a molecular beacon further comprises complementary 5' end and 3' end flanking regions, which are labeled with a fluorescent dye in the 5′ end and a quencher molecule in the 3′ end. In theory, all fluorophore quencher pairs are suitable for use as molecular beacon probes. Suitable combinations of 5' fluorescent reporter dyes and 3' quenchers include, but are not limited to, 5' 6-FAM with either 3' Dabcyl, 3' Black Hole Quencher® 1 (BHQ1, Biosearch Technologies, Inc.), or 3' Iowa Black® FQ (3IABkFQ, Integrated DNA Technologies, Inc.); 5' HEX with 3IABkFQ; 5' TET™ (Integrated DNA Technologies, Inc.) with 3IABkFQ; 5' TYE™ 563 with 3' Iowa Black® RQ-Sp (both Integrated DNA Technologies, Inc.), and 5' TYE™ 665 (Integrated DNA Technologies, Inc.) with 3' Iowa Black® RQ-Sp.

In still other preferred methods of the present invention, the reverse transcription and loop-mediated isothermal amplification reactions are performed in a single reaction mixture comprising template RNA, dNTP's, a thermostable reverse transcriptase, and a DNA polymerase having strand displacement activity, more preferably (Bst) DNA polymerase.

In still other preferred methods of the present invention, the consensus sequence is a consensus sequence as set forth in any one of Figures 10-15 and sequences having at least 80% sequence identity thereto.

In another aspect, the present invention provides a method for the detection of inducible HIV-1 in a subject receiving or having received antiretroviral therapy (ART), the method comprising the steps of:

a) providing a sample of $CD4^+$ T cells from said subject;

b) stimulating said $CD4^+$ T cells in said sample to thereby provide a sample of activated $CD4^+$ T cells;

c) performing the method of any one of claims 1-8 on said sample of activated $CD4^+$ T cells, and detecting an amplification product of the RT-LAMP reaction as an indication of the presence of inducible HIV-1 in said sample or in said subject.

The step b) of stimulating said $CD4^+$ T cells in said sample to thereby provide a sample of activated $CD4^+$ T cells is incorporated to activate latent HIV-1 in said cells. Stimulating said $CD4^+$ T cells in order to activate latent HIV-1 therein is well known in the art, and generally comprises exposing said cells to an effective amount of a latency-reversing compound. Examples of such compounds include, but are not limited to a compound selected from anti-CD3 antibody, anti-CD28 antibody, anti-histone deacetylase (HDAC) antibody, apicidin, 5-azacytidine (5-Aza), 5-Aza-CdR, azelaic bishydroxamic acid (ABHA), bryostatin, butyric acid, m-carboxycinnamic acid bis-hydroxamide (CBHA), dacinostat (LAQ824), 12-deoxyphorbol 13-phenylacetate (DPP) depudecin, entinostat (MS-275), 5-fluoro-2'-deoxycytidine, glutamate, hexamethylbisacetamide (HMBA), hydralazine, IL-7, IL-2, IL-1ß, ingenol mebutate, 3-caproyl-ingenol, ingenol 3,20-dibenzoate, ionomycin, M344 (D237), midostaurin (PKC412), oxamflatin, panobinostat (LBH589), phenylbutyrate, phenylacetate, phorbol myristate acetate (PMA), N-phthalyl-L-tryptophan (RG108), phytohemagglutinin (PHA), procaine, procainamide, prostratin, pyroxamide, romidepsin (FK228), scriptaid, sodium butyrate, suberic bishydroxamic acid (SBHA), suberoylanilide hydroxamic acid (SAHA), tacedinaline (CI-994), trapoxin B, trichostatin A, tumor necrosis factor-α (TNFα), valproic acid, zebularine, and combinations thereof.

In another aspect, the present invention provides a method of quantifying the inducible HIV-1 reservoir in a subject receiving or having received antiretroviral therapy (ART), the method comprises performing the

method for the detection of inducible HIV-1 in a subject receiving or having received antiretroviral therapy (ART) as described above, wherein said step b) of said method further comprises preparing serial volumetric dilutions of said sample of activated $CD4^+$ T cells, and wherein said step c) is followed by the step of quantifying the amount of Tat/Rev multiply spliced HIV-1 RNA-containing $CD4^+$ T cells in said sample as a ratio of the total number of $CD4^+$ T cells in the sample provided in said step a) to thereby provide an indication of the size of the inducible HIV-1 reservoir in said subject. It will be understood that the amount of Tat/Rev multiply spliced HIV-1 RNA-containing $CD4^+$ T cells in said sample may depend on the number of Tat/Rev multiply spliced HIV-1 RNA copies in said cell and the sensitivity of the assay, and may therefore also be indicated as the amount of detectable Tat/Rev multiply spliced HIV-1 RNA-containing $CD4^+$ T cells.

In an alternative embodiment of a method of quantifying the inducible HIV-1 reservoir in a subject receiving or having received antiretroviral therapy (ART) in accordance with the present invention, the method comprises performing the method for the detection of inducible HIV-1 in a subject receiving or having received antiretroviral therapy (ART) as described above directly on individual $CD4^+$ T cells in or from said sample of activated $CD4^+$ T cells provided in said step b), and wherein said step c) is followed by the step of quantifying the amount of (detectable) Tat/Rev multiply spliced HIV-1 RNA-containing $CD4^+$ T cells in said sample as a ratio of the total number of $CD4^+$ T cells in the sample provided in said step a) to thereby provide an indication of the size of the inducible HIV-1 reservoir in said subject.

When a method for the detection of inducible HIV-1 in a subject receiving or having received antiretroviral therapy (ART) as described above is performed directly on individual $CD4^+$ T cells, it is preferred that the amplification product of the RT-LAMP reaction is detectable by flow cytometry, such as by fluorescence-activated cell sorting (FACS). Thereto the primer set preferably comprises an LF or LB loop primer, wherein said

LF or LB loop primer sequence is the target-specific sequence for a probe or molecular beacon that is labeled with a fluorescent dye (e.g. in the 5′ end) and a quencher molecule (e.g. in the 3′ end). This will result in a molecular beacon that is detectable in individual cells.

A method of the present invention may employ limiting dilution and maximum likelihood estimation to detect and quantify the HIV-1 msRNA within the latent reservoir. In this method, cells from people living with HIV (PWH) are serially diluted and subjected to isothermal loop amplification as described above to detect the presence of HIV-1 msRNA. The results are then analyzed using maximum likelihood estimation to determine the frequency of cells containing inducible HIV-1. This approach allows for the detection of latent HIV-1, and provides crucial insights into the reservoir size and potential reactivation under antiretroviral therapy (ART).

In a further embodiment of a method of the invention, use can be made of quantification by single cell approach, also referred to herein as single-cell SQuHIVLa. Single cell quantitation allows accurate quantitation of smaller reservoirs, especially post-intervention or in individuals with naturally small reservoirs. Moreover, such single cell assays, wherein the HIV-1 msRNA is amplified in individual cells in situ, suffer less from background signals due to combined total RNA.

In a further embodiment of a method of the invention, the method of detecting an amplification product in a sample, comprising performing an RT-LAMP reaction with said sample with a primer set specific for Tat/Rev multiply spliced HIV-1 RNA and determining whether the sample comprises an amplification product of the amplification reaction as described above, is performed directly on whole cells, such as PBMCs, preferably isolated CD4+ T cells. This method enables the detection of HIV-1 msRNA in individual cells. Cells comprising amplified Tat/Rev multiply spliced HIV-1 RNA can be quantified by flow cytometry, allowing for detailed analysis of cellular

subsets, providing more granular insights into the characteristics of the latent reservoir.

Thus in a preferred embodiment of the invention, the methods of the invention are performed on whole cells, preferably on activated CD4+ T cells. When using whole cells, such cells are preferably fixed to withstand lysis during the amplification procedure. The amplification product is then readily detectable in such whole single cells, for instance by using a molecular beacon as described herein in combination with flow cytometry. Preferably, in whole single cells methods of this invention, the cells are fixed and or permeabilized. A suitable procedure for fixation and permeabilization involves treating the cells with methanol. Other methods of cell fixation and permeabilization are envisioned and are well known in the art.

The invention further provides in another aspect, the use of a set of primers for amplifying Tat/Rev ms HIV-1 DNA from HIV-1 subtype B comprising:

- a forward outer primer having the sequence GTGTTGCTTTCATTGCCAAG;
- a backward outer primer having the sequence GTCTCTCTCTCCACCTTCT;
- a forward inner primer having the sequence TGAGGAGCTCTTCGTCGCTGCAAAAGCCTTAGGCATCTC;
- a backward inner primer having the sequence CAGTCAGACTCATCAAGTTTCTCTCTTCGATTCCTTCGGGCC;
- a forward loop primer having the sequence TCTCCGCTTCTTCCTGC; and
- a backward loop primer having the sequence AACCCACCTCCCAACCC.

The invention further provides in another aspect, the use of a set of primers for amplifying Tat/Rev ms HIV-1 DNA from HIV-1 subtype C comprising:

- a forward outer primer having the sequence TCCTTGTAATAAGTGTTATTGTAA;
- a backward outer primer having the sequence CGATTCTTCCGAGCCTGTC;
- a forward inner primer having the sequence CCGCTTCTTCCTGCCATAGGAATAGCTATCATTGTCTAGTTTGC;
- a backward inner primer having the sequence CGACGAAGCGCTCCTCCAAGGTTTGGGGTAAGGGTTGCT;
- a forward loop primer having the sequence TGCCTAAGCCTTTTGTCTG, and
- a backward loop primer having the sequence CAGTGAGGATCATCAAAATC.

The person skilled in the art will understand that in addition to the above primer and probe sequences, primer and probe sequences that have at least 75%, preferably at least 80%, preferably at least 85%, preferably at least 90%, preferably at least 95% and more preferably at least 98% sequence identity with the above specified sequences are equally suitable for use in aspects of this invention.

The invention further provides a kit of parts for use in a method of the invention *in situ*, comprising said primer set specific for Tat/Rev multiply spliced (ms) HIV-1 RNA amplification by a reverse transcription, loop-mediated isothermal amplification (RT-LAMP) reaction and optionally dNTP's, a thermostable reverse transcriptase, a DNA polymerase having strand displacement activity, more preferably (Bst) DNA polymerase, and - a suitable buffer, preferably an isothermal amplification buffer in one or more containers.

In preferred embodiments of a kit of parts of the invention, the reagents may be pre-mixed provided the individual reagents in the mixture can be stored, either at room temperature of in frozen or freeze-dried form, and provided the reagents are stable in the mixture in that they retain their

activity during storage. In another preferred embodiment, each reagent is contained in a separate container.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Flow chart of sequential steps in designing Tat/Rev msRNA LAMP primer/probe set.

Figure 2. Schematic overview of SQuHIVLa assay and primer binding sites specific for HIV-1 subtype B. Panel A) Schematic overview of the primer binding regions where the splice site is located within the forward or backward loop. Primers bound to msRNA results in a stable forward and backward loop formation. However, the presence of intron (in case of Tat/Rev DNA or unspliced RNA transcript) leads to an unstable forward or backward loop formation, depending on the position of the intron within 5'F2 to 3'F1 or 5'B2 to 3'B1 respectively. Panel B) Schematic overview of HIV-1 genome and transcripts produced during the HIV-1 replication cycle and alignment of the different LAMP primer binding regions with Ten HIV-1 subtype B viral sequences. The solid line represents the exons and the dotted lines represent the intron that is excised to generate mature HIV-1 mRNA transcripts. Exons of a multiply spliced RNA transcripts have colored rectangles that represent approximate LAMP primer binding sites. Similar to the alignment, different colors correspond to different regions of the primers. After alignment, the "*" symbol is used to denote identical nucleotides; otherwise, the actual nucleotide symbol (A, T, C, or G) is used.

Figure 3. Determination of the sensitivity of RT-LAMP used in SQuHIVLa assay in detecting msRNA. Panel A) Experimental outline to generate synthetic msRNA using T7 polymerase mediated by in vitro transcription. Synthetic msRNA is serially diluted to achieve samples containing a range of target RNA copies used to perform RT-LAMP. Panel B) Percentage of positive RT-LAMP reactions and Panel C) amplification time required for a range of RNA copies are plotted. Data are presented as

mean ± SD of three independent in vitro transcription experiments followed by serial dilution and RT-LAMP. Panel D) A non-linear regression analysis is performed using the proportion of positive RT-LAMP reactions (Hit rate) (Y-axis) corresponding to Log10 values of RNA copies used as a template (X-axis). Panel E) Probit analysis is performed to determine LOD-95% using probit values calculated from HIT-rate (Y-axis) corresponding to Log10 values of RNA copies (Y-axis). Panel F) Experimental outline to sort single GFP+ J-Lat 11.1 cell in each well of 96 well PCR plate containing either no cells or different number of stimulated healthy donor CD4+ T cells to determine the sensitivity of RT-LAMP. Panel G) Percentage of RT-LAMP positive reactions when a single GFP+ J-Lat 11.1 cell/reaction is probed in the absence of cellular background. Data are presented as mean ± SD of four independent experiments and each experiment is represented with different colored dots. H) Percentages of RT-LAMP positive reactions when a single GFP+ J-Lat 11.1 cell/reaction is probed in the presence of an increasing background of uninfected donor CD4+T cells and their corresponding RT-LAMP duration. Data are presented as mean ± SD of three independent experiments. I) Ordinary one-way ANOVA followed by Dunnett's multiple comparison test are performed to analyze the variation of amplification times among different conditions and statistical significance is determined by $p < 0.05$.

Figure 4. Quantification of inducible HIV-1 reservoir using SQuHIVLa assay. Panel A) Percentages of RT-LAMP positive reactions when a single GFP+ J-Lat 11.1 cell/reaction was probed with or without reverse transcriptase enzyme included in the RT-LAMP reaction, and when either a single GFP+ or GFP- J-Lat 11.1 cell/reaction was probed. Panel B) Percentages of positive reactions when a DNase-treated RNA and RNase-treated DNA sample isolated from PMA-stimulated J-Lat 11.1 cells, or when pNL4.3 E-R- plasmid were used as template for RT-LAMP. Panel C) Experimental outline of preparing custom samples with an inducible HIV-1 reservoir of 0.1, 1, 5, 20 GFP+ cells/millions of CD4+ T cells using

stimulated J-Lat 11.1 cells and uninfected donor CD4+ T cells. These custom samples were used to quantify the reservoir size using the SQuHIVLa assay. Panel D) The inter assay coefficient of variation (CV) is determined from four independent experiments for all four samples representing different inducible reservoir size (0.1, 1, 5, 20 GFP+ cells/million CD4+ T cells). Four different colored-symbols represent data from four individual experiments. Panel E) The Pearson correlation coefficient (r2) is determined between expected and experimental inducible reservoir size and statistical significance is determined by $p < 0.05$. The accuracy percentage (AP) was calculated with Excel using ABS function. Panel F) A brief experimental outline of the application of SQuHIVLa to quantify inducible HIV-1 subtype B reservoir using PMA/ionomycin-activated CD4+ T cells in limited dilution format followed by maximum likelihood calculation. Panel G) The inducible HIV-1 reservoir was quantified for three different people living with HIV-subtype B (PLWHB) using a SQuHIVLa protocol adapted to include an incubation at 45°C prior to RT-LAMP in order to determine the optimal incubation time for primary samples. Three different colored symbols represent data of three PLWHB. Panel H) The inter assay CV is determined from three independent experiments for three PLWHB. Data are presented as mean ± SD and three different colored symbols represent three PLWHB.

Figure 5. Design and application of SQuHIVLa assay to quantify inducible reservoir of People Living with HIV-1 subtype C. Panel A) Ten HIV-1 subtype C viral sequences are aligned with different subtype C specific LAMP primers. After alignment, the "*" symbol is used to denote identical nucleotides; otherwise, the actual nucleotide symbol (A, T, C, or G) is used. In vitro transcribed synthetic subtype C msRNA is serially diluted to achieve samples containing a range of target RNA copies used to perform RT-LAMP with subtype C specific primers and probe. Panel B) Percentage of positive RT-LAMP reactions and Panel C) amplification time required for different amounts of RNA copies are plotted. Data are presented as mean ±

SD of three independent in vitro transcription experiments followed by serial dilution and RT-LAMP. Panel D) A non-linear regression analysis is performed using proportion of positive RT-LAMP reactions (HIT rate) (Y-axis) corresponding to Log10 values of RNA copies used as a template (X-axis). Panel E) Probit analysis is performed to determine LOD-95% using probit values calculated from the HIT-rate (Y-axis) corresponding to Log10 values of RNA copies (Y-axis). Panel F) The inducible HIV-1 reservoir was quantified for N=19 people living with HIV-1 subtype C (PLWHC) presented as median with range. Panel G) The inducible HIV-1 reservoir was quantified for ten female (blue open circle) and seven male (red square) PLWHC using SQuHIVLa assay. Data are presented as mean ± SD. Two-tailed unpaired t-test is performed to analyze the variation of inducible HIV-1 reservoir between male and female PLWHC and statistical significance is determined by $p < 0.05$.

Figure 6 (Suppl. Figure 1). Tools used for evaluating RT-LAMP sensitivity. A) Percentage of positive LAMP reactions and B) amplification time required for different amount of RNA copies are plotted when the amplification was carried out without reverse transcriptase enzyme. Data are presented as mean ± SD of three independent reactions, each of which had four technical replicates. C) Schematic overview of the integrated HIV-1 genome in JLat 11.1 cells. The green rectangle depicts the substitution of the Nef gene with GFP in the integrated HIV-1 genome, and the red rectangle represents the mutation in the HIV-1 Env gene that results in a faulty viral envelope protein. D) Sorting of GFP+ positive JLat 11.1 cells upon PMA stimulation. GFP-positive cells are represented by the green cell population, and GFP-negative cells by the purple cell population.

Figure 7 (Suppl. Figure 2). RT-LAMP for the precise detection of msRNA. A) Amplification time required for RT-LAMP when a single GFP+ J-Lat 11.1 cell/reaction were used as input for RT-LAMP amplification with or without reverse transcriptase enzyme B) Amplification time required for RT-LAMP when a single GFP+ or GFP- J-Lat-11.1 cell/reaction were used

as input for amplification. Data are presented as mean ± SD of three independent experiments, each of which had 15 technical replicates.

Figure 8 (Suppl. Figure 3). Heat map of the mismatches of subtype B Tat/Rev Primers binding to HIV-1 subtype B (A), C (B) and A (C). Y-axis represents different primers and x-axis represents nucleotide positions. The value, and corresponding color, in the heat map portrays the relative number of mismatches that occur at each position.

Figure 9 (Suppl. Figure 4). Correlation of inducible reservoir size of male and female participants quantified by SQUHIVLA with different clinical parameters. The Pearson correlation coefficient (r2) is determined between inducible reservoir size quantified using SQuHIVLa and CD4 nadir for 10 female participants (A) and 7 male participants (B). The Pearson correlation coefficient (r2) is also determined between inducible reservoir size quantified using SQuHIVLa and CD4+ T cell count at the time of sampling for 10 female participants (C) and for 7 male participants (D). Female participants are depicted with blue open circle and male participants are depicted with red square. Statistical significance is determined by $p < 0.05$.

Figure 10. Exemplary HIV-1 subtype B Tat/Rev ms HIV-1 RNA consensus sequence (490 bp). The sequence of each subtype is split into two paragraphs exactly at 215th nucleotide (splice site); The start and end position of the target region (5'F2 to 3'B2c) from nucleotide position 55 to 375 are pointed out in bold typeface and larger font size. Only F2 and B2c primers within this target region can be considered for the final selection; preferred F2 binding region is underlined; preferred B2 binding region is indicated by letters in italics typeface (cursive font).

Figure 11. Exemplary HIV-1 subtype C Tat/Rev ms HIV-1 RNA consensus sequence (463 bp). Idem as Fig. 10.

Figure 12. Exemplary HIV-1 subtype A Tat/Rev ms HIV-1 RNA consensus sequence (490 bp). Idem as Fig. 10.

Figure 13. Exemplary HIV-1 subtype D Tat/Rev ms HIV-1 RNA consensus sequence (490 bp). Idem as Fig. 10.

Figure 14. Exemplary HIV-1 subtype AE Tat/Rev ms HIV-1 RNA consensus sequence (511 bp). Idem as Fig. 10.

Figure 15. Exemplary HIV-1 subtype AG Tat/Rev ms HIV-1 RNA consensus sequence (491 bp). Idem as Fig. 10.

Figure 16. Result of single-cell SQuHIVLa (sc-SQuHIVLa) as described in Example 5. In this figure, the X-axis represents the fluorescence of the SQuHIVLa-specific FAM fluorophore, and the Y-axis represents the fluorescence of a Live/Dead cell staining. Q1: Dead cell that are Fam negative; Q2: Dead cells that are FAM positive due to autofluorescence; Q3: Live cells that are FAM positive due to SQuHIVLa signal; Q4: Live cells that are FAM negative. YGA 780_60: for viability dye (exc: 633 nm; em: 780 nm); BB 530_30: for 6-FAM (exc. 488 nm; em. 518 nm).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the term "human immunodeficiency virus", abbreviated "HIV" refers to a Lentivirus (a subgroup of retrovirus) that infects humans and causes acquired immunodeficiency syndrome (AIDS), a condition that results in progressive failure of the immune system. HIV is divided into two types, HIV-1 and HIV-2, among which HIV-1 is the most common. HIV-1 is further separated into three groups: major Group M (major), non-major Group N (non-M, non-O), and Group O (outlier). By far, more than 90% of AIDS cases derive from the infection of HIV-1 Group M. Group M is divided into subtypes (clades), among these are the most studied subtypes: A, B, C, and D. The term "human immunodeficiency virus-1" (HIV-1) includes reference to any of a variety of HIV-1 subtypes.

The term "CD4+ T cell", as used herein refers to a T cell which expresses the surface protein CD4. In an embodiment, the T cell expresses

the surface protein CD4 at a sufficient level to be detected, e.g., by flow cytometry. The term includes reference to resting $CD4^+$ T cells and total $CD4^+$ T cell population, preferably resting CD4+ T cells. CD4+ T cells can be isolated by negative selection from Peripheral blood mononuclear cells (PBMCs) using commercial enrichment kits, such as the EasySep Human CD4+ T Cell Enrichment Kit (STEMCELL Technologies), wherein non-CD4+ T cells are bound to magnetic particles and removed. PBMCs may for instance be isolated from blood and leukapheresis samples using Ficoll-Hypaque gradients as known in the art. Resting CD4+ T cells may be isolated from the CD4+ T cell preparations by negative selection of cells expressing CD25, CD69, and HLA-DR (e.g., CD25 MicroBeads II, 130-092-983; CD69 MicroBead Kit II, 130-092-355; anti–HLA-DR MicroBeads, 130-046-101; Miltenyi Biotec). PBMCs may be viably frozen and thawed before CD4+ T isolation.

As used herein, the term "loop-mediated isothermal amplification", abbreviated "LAMP", refers to the DNA amplification process as described in, for instance, Notomi et al., *Nucl. Acids Res.* 28:E63 (2000), US6,410,2778, US20080182312A1, EP1020534A1 and EP1327679A1. In brief, Figure 1 of US20080182312A1 provides a schematic representation of the loop-mediated isothermal amplification (LAMP) of nucleic acids. Figure 1a of US20080182312A1 describes the generation of the Loopamp Starting Structure. Step 1, forward inner primer region 'F2' binds to complementary sequence on the target sequence. The polymerase initiates primer extension while displacing the target complimentary strand. Step 2, polymerase completes copy of target sequence. Step 3, the 'F3' primer binds to complementary sequence on the target sequence and polymerase initiates primer extension. Step 4, primer extension from the 'F3' primer displaces forward inner primer product. The 'F1c' and 'F1' on the displaced forward inner primer product hybridize to form a hairpin loop. Step 5, backward inner primer region 'B2' binds to complementary sequence on the displaced product. The polymerase initiates primer extension. Step 6, polymerase

displaces hairpin and completes primer extension. Step 7, the 'B3' primer binds to complementary sequence and primer extension is initiated. Step 8, primer extension completely displaces a single strand product that forms hairpin loops at each end. This is the starting structure for the amplification phase of the Loopamp. Primer extension beginning at the forward inner primer site is shown in Figure 1 of US20080182312A1 as a representative initiation of the process - the process can initiate at either the forward inner primer site or backward inner primer site. Figure 1b of US20080182312A1 describes the amplification of Loopamp Starting Structure. Forward inner primer and backward inner primer bind to complementary sequences on the Loopamp starting structure and initiate primer extension and strand displacement by the polymerase. Continued hybridization of the forward inner primer and backward inner primer followed by primer extension and strand displacement results in the formation of products of different lengths and generation of more Loopamp starting structures. In preferred embodiments of methods of the present invention, the polymerase enzyme is *Bacillus stearothermophilus* (Bst) DNA polymerase. If the target is RNA, the target may be converted into cDNA using a reverse transcriptase enzyme

The term "sequence identity" as used herein refers to sequences which in their full length sequence display at least an indicated % of identity to the full length of the sequence to which the identity is indicated. In practice, this means that the identity has to be calculated as the number of identical residues in a global alignment of the two sequences over the number of residues of the longer of the two sequences. The term "sequence identity" as used herein refers to the extent that sequences are identical on a nucleotide-by-nucleotide basis over a window of comparison. Thus, a "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T/U, C, G) occurs in both sequences to yield the number of matched positions, dividing

the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. A number of different computer programs can be used to identify whether a polynucleotide has sequence identity or similarity to a known sequence. Sequence identity or similarity may be determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman, Adv. Appl. Math. 2: 482 (1981), by the sequence identity alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48: 443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. USA 55:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), the Best Fit sequence program described by Devereux et al, Nucl. Acid Res. 72:387 (1984), preferably using the default settings, or by inspection.

As used herein, the term "*tat/rev* multiply spliced (ms) RNA" refers to a particular splicing profile of the HIV-1 primary transcript. The splicing profile of the HIV-1 primary transcript is highly complex, regulated by four splice donors and eight splice acceptors and several additional cryptic, cis, and trans-regulatory elements. Further, strain differences, subtype-specific variations, and host cell lineage and activation differences can add more complexity to the splicing of the viral transcript. Approximately 40-60 differently spliced transcripts classified into three broad categories may be identified intracellularly: un-spliced, partially spliced, and completely spliced transcripts. The completely spliced viral transcripts of approximately 1.8 kb code for the three early viral proteins: Tat, Rev and, Nef. Low amounts of cell-associated unspliced HIV RNA are frequently detected in PBMCs from virally suppressed subjects on antiretroviral therapy (ART), as well as in latently infected $CD4^+$ T cells that do not produce HIV particles, and therefore, cannot be used as a

surrogate for viral replication. In contrast, tat/rev multiply spliced (ms) HIV-1 RNA better reflects HIV-1 viral replication-competence.

As used herein, the term "consensus sequence" refers to a sequence that is common to, or otherwise present in the largest fraction, of an aligned group of sequences. The consensus sequence shows the nucleotide most commonly found at each position within the nucleic acid sequences of the group of sequences.

As used herein, the term “conserved nucleotide” refers to nucleotides in a sequence alignment that appear to mutate slower than nucleotides at other positions in the sequence. Conserved nucleotides can be identified by visual comparison or by computer analysis of a multiple sequence alignment, and determining the frequency of occurrence of a specific nucleotide residue at each position within said alignment, wherein nucleotide positions that show a common nucleotide or that statistically show to contain a specific residue with a frequency that is higher than that of other positions among the is indicated as a conserved position, and its corresponding nucleotide as a conserved nucleotide.

As used herein, the term “oligonucleotide” refers to a short polymer composed of deoxyribonucleotides, ribonucleotides or any combination thereof. Oligonucleotides of the invention are at least 10 but not more than 100 nucleotides in length. Oligonucleotides are preferably 20 to 70 nucleotides long, with 21 to 26 nucleotides being the most common. An oligonucleotide may be used as a primer or as a probe.

As used herein, the term "self-quenching probe" refers to an oligonucleotide that is labeled with a reporter and a quencher such that when the probe is not hybridized to other sequences, the quencher absorbs the reporter signal and when the probe hybridizes to its complement, the quencher is unable to absorb the reporter signal and the probe is detectable.

The term “molecular beacon”, as used herein, refers to a dye-labeled oligonucleotide probe (25–40 nt) that forms a hairpin structure with a stem and a loop. The 5' and 3' ends of the probe have complementary

sequences of 5–6 nucleotides that form the stem structure. The loop portion of the hairpin is designed to specifically hybridize to a 15–30 nucleotide section of the target sequence. A fluorescent reporter molecule is attached to the 5' end of the molecular beacon, and a quencher is attached to the 3' end or in close proximity of those positions. Formation of the hairpin brings the reporter and quencher together, so no fluorescence is emitted. During the annealing step of the amplification reaction, the loop portion of the molecular beacon binds to its target sequence, causing the stem to denature. The reporter and quencher are thus separated, quenching is abolished, and the reporter fluorescence is detectable. Because fluorescence is emitted from the probe only when it is bound to the target, the amount of fluorescence detected is proportional to the amount of target in the reaction. Unlike hydrolysis probes, molecular beacons are displaced but not destroyed during amplification due to the use of a DNA polymerase lacking 5' exonuclease activity. Reporter and quencher pairs can be any of the commonly used fluorescent reporters 6-FAM (520nm), TET (539nm), YAK (Yakima Yellow®, 549nm), VIC (554), SUN (554nm), HEX (555nm), or JOE (555nm), in combination with quenchers TAMRA, Black Hole Quencher 1 (BHQ®-1) or Iowa Black FQ (single-quenched) or ZEN-Iowa Black FQ (double-quenched); or fluorescent reporters Cy®3 (564nm), ATTO ™ 550 (575nm), TAMRA (583), ATTO 565 (591nm), PET® (595nm), ROX (608nm), Texas Red®-X (617nm), JUN® (617nm), ATTO 633 (657nm), LIZ® (655nm), Cy5 (668nm), and ATTO 647 (669nm) in combination with quenchers Black Hole Quencher-2 (BHQ-2), Iowa Black RQ (single-quenched), or TAO–Iowa Black RQ (double-quenched).

As used herein, an oligonucleotide is “specific” for a nucleic acid if the oligonucleotide has at least 50% sequence identity with a portion of the nucleic acid when the oligonucleotide and the nucleic acid are aligned. An oligonucleotide that is specific for a nucleic acid is one that, under the appropriate hybridization or washing conditions, is capable of hybridizing to the target of interest and not substantially hybridizing to nucleic acids

which are not of interest. Higher levels of sequence identity are preferred and include at least 75%, preferably at least 80%, preferably at least 85%, preferably at least 90%, preferably at least 95% and more preferably at least 98% sequence identity.

The term "primer" refers to an oligonucleotide, generally produced synthetically, that is capable of acting as a point of initiation of polynucleotide synthesis when placed under conditions in which synthesis of a primer extension product that is complementary to a nucleic acid strand is induced, (e.g., in the presence of nucleotides and polymerase or reverse transcriptase enzyme and at a suitable temperature and pH). The primer is preferably single stranded. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the polymerase or reverse transcriptase enzyme. The exact lengths of the primers will depend on many factors, including temperature used in the amplification method.

The length of the amplification primers for use in the present invention depends on several factors including the nucleotide sequence identity and the temperature at which these nucleic acids are hybridized or used during in vitro nucleic acid amplification. The considerations necessary to determine a preferred length for an amplification primer of a particular sequence identity are well known to the person of ordinary skill. For example, the length of a short nucleic acid or oligonucleotide can relate to its hybridization specificity or selectivity.

A primer for reverse transcription and/or amplification such as by LAMP is an oligonucleotide that specifically anneals to a target nucleotide sequence. The three most 3′ nucleotides of the primer are preferable identical to the target sequence at a corresponding nucleotide position.

The term "reverse transcriptase" describes a class of polymerases characterized as RNA-dependent DNA polymerases. All known reverse transcriptases require a primer to synthesize a DNA transcript from an RNA template. Reverse transcriptase may be used to transcribe RNA into

cDNA. The invention contemplates the use of any suitable reverse transcriptase. Preferably the reverse transcriptase is a thermostable reverse transcriptase, preferably a thermostable reverse transcriptase that is reversibly inhibited at room temperature and is activated when warmed above about 40°C. Preferably having a maximum enzymatic activity at about 55°C.

As used herein, the term "reverse transcription" indicates the capability of enzyme to synthesize DNA strand (that is, complementary DNA or cDNA) using RNA as a template.

The term "cDNA" refers to a strand of DNA copied from an RNA template. cDNA is complementary to the RNA template.

As used herein, a “forward primer” is a primer that anneals to the anti-sense strand of dsDNA. A “reverse primer” anneals to the sense-strand of dsDNA.

As used herein, the term “reaction mixture” means any mixture that includes all the components required to synthesize a DNA copy of an RNA target molecule and to amplify the resulting cDNA by isothermal amplification, preferably by RT-LAMP. Typically, a “reaction mixture” includes reverse transcriptase enzyme and DNA polymerase as indicated herein and dNTP's (also referred to as nucleotides herein) in a suitable buffer. A suitable buffer in aspects of this invention may also, and preferably, refer to an isothermal amplification buffer for use in a RT-LAMP amplification and may typically include: (a) a buffering agent such as Tris to maintain a pH of about 8; (b) salt such as KCl; and (c) a source of magnesium ions such as $MgCl_2$. Such isothermal amplification buffers are commercially available from a variety of vendors. The reaction mixture may be prepared to include all reagents of the RT-LAMP reaction, and the reaction may be started by addition of the RNA target, e.g. in the form of a purified or crude sample of a subject, preferably a sample believed to comprise HIV-1 nucleic acids, more preferably comprising $CD4^+$ T cells of a

subject receiving or having received ART treatment, and bringing the reaction mixture to the appropriate temperature for amplification.

As used herein, the term "hybridize" refers to a process that two complementary nucleic acid strands anneal to each other under appropriately stringent conditions. Hybridizations are typically and preferably conducted with oligonucleotides, preferably 20-100 nucleotides in length. Nucleic acid hybridization techniques are well known in the art. See, e.g., Sambrook, et al., 1989, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press, Plainview, N.Y. Those skilled in the art understand how to estimate and adjust the stringency of hybridization conditions such that sequences having at least a desired level of complementarity will stably hybridize, while those having lower complementarity will not. For examples of hybridization conditions and parameters, see, e.g., Sambrook, et al., 1989, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press, Plainview, N.Y.; Ausubel, F. M. et al. 1994, Current Protocols in Molecular Biology. John Wiley & Sons, Secaucus, N.J.

The term "stringent hybridization conditions" as used herein refers to hybridization conditions at least as stringent as the following: hybridization in 50% formamide, 5×SSC, 50 mM NaH2PO4, pH 6.8, 0.5% SDS, 0.1 mg/mL sonicated salmon sperm DNA, and 5×Denhardt's solution at 42° C. overnight; washing with 2×SSC, 0.1% SDS at 45° C.; and washing with 0.2×SSC, 0.1% SDS at 45° C. In another example, stringent hybridization conditions should not allow for hybridization of two nucleic acids which differ over a stretch of 20 contiguous nucleotides by more than two bases.

The term "amplify" with respect to nucleic acid sequences refers to methods that increase the representation of a population of nucleic acid sequences in a sample. Nucleic acid amplification methods, such as LAMP are well known to the skilled artisan.

As used herein, the term "sample" refers to any liquid or solid material believed to comprise HIV-1 nucleic acids. In preferred embodiments, a sample is obtained from a biological source, such as cells in culture or a tissue sample or body fluid from an animal, most preferably, a human. Preferred sample tissues or body fluids include, but are not limited to, plasma, serum, whole blood, blood cells, lymphatic fluid, cerebrospinal fluid, synovial fluid, urine, saliva, and skin or other organs (e.g. biopsy material). The term "patient sample" as used herein refers to a sample obtained from a human seeking diagnosis or treatment for HIV-1 infection.

It is to be noted that strategies toward HIV cure aim to eliminate, inactivate, reduce or immunologically control the pool of latently infected cells such that combination antiretroviral therapy (cART) can be safely interrupted. In order to assess the impact of any putative curative interventions on the size and inducibility of the latent HIV-1 reservoir, robust and scalable assays are needed to precisely quantify the frequency of infected cells containing inducible replication competent HIV-1. The present invention provides methods for Specific Quantification of Inducible HIV by LAMP (SQuHIVLa). SQuHIVLa presents a novel assay that leverages the high sensitivity and specificity of RT-LAMP, performed in a single reaction, to detect and quantify cells expressing Tat/Rev msRNA upon activation. The LAMP primer/probes used in methods of the present invention exclusively detect subtype-specific HIV-1 Tat/Rev ms RNA and exhibit high sensitivity, specificity, and reproducibility. Using SQuHIVLa, the present inventors were able to quantify the inducible viral reservoir in CD4+ T cells from a diverse group of individuals with HIV-1 subtypes B and C on suppressive antiretroviral therapy. SQuHIVLa presents a high throughput, scalable and specific HIV-1 reservoir quantification tool that is amenable to resource limited settings.

Combination antiretroviral therapy (cART) has changed human immunodeficiency virus type -1 (HIV-1) infection from a lethal to a chronic

illness. However, despite over 40 years of research, there is currently no safe, scalable, curative therapy in sight. Furthermore, HIV-1 treatment disparities persist, with approximately 70% of all individuals with HIV-1 residing in sub-Saharan Africa, and only 78% with access to treatment[2]. While standard clinical assays have shown that cART can effectively reduce circulating virus in the blood to undetectable levels, HIV-1 still persists in the form of an integrated provirus within a subset of immune cells, particularly resting memory CD4+ T cells (the viral reservoir). If suppressive cART is interrupted, inducible replication-competent HIV-1 provirus (the inducible viral reservoir) can reactivate and exponentially replicate leading to detectable levels of viremia (viral rebound), within two weeks, in a majority of individuals[5]. Lifelong therapy is thus necessary to maintain viral suppression, and interventions beyond cART are urgently needed to achieve an HIV-1 cure.

Toward this aim, several strategies that target the inducible viral reservoir for elimination or control are being explored. To understand HIV-1 reservoir persistence, reliable biomarkers of reservoir size and dynamics are needed to evaluate the potential impact of interventions that aim to reduce the size of the inducible viral reservoir. Furthermore, robust, precise, and high throughput and scalable assays capable of measuring the frequency of infected cells containing inducible replication-competent HIV-1 are required. However, the low frequency of these cells, estimated at between 1 and 1000 per million CD4+ T cells makes quantification extremely challenging, highlighting the need for innovative solutions to identify, target and quantify the viral reservoir in the quest for an HIV-1 cure.

Numerous techniques have been proposed, developed, or used to measure the HIV-1 reservoir. PCR-based assays offer a practical method to determine the frequency of cells containing total or integrated HIV-1 DNA. However, these assays considerably overestimate the size of the viral reservoir. By targeting only one highly conserved proviral region, defective HIV-1 genomes that harbor large deletions or hypermutations in regions

external to the assay amplicon are inclusively detected. Recent technological advances have addressed this issue by introducing assays that enable the detection of more than one conserved region of the HIV-1 genome. The intact proviral DNA assay (IPDA), for example, targets the packaging signal (PSI) and *env*-RRE regions using digital droplet (dd)PCR technology. Although successful in excluding the majority of defective viruses, conventional IPDA targets a very small sub-genomic region (2% of the HIV-1 genome), which can lead to an overestimation of the viral reservoir. Adapted multiplex versions of IPDA have integrated additional assay targets for regions in HIV-1 *gag* and *pol* genes. A comparable technique to assess HIV-1 genome integrity, called Q4PCR, used a combination of a quadruplex qPCR and next-generation sequencing approach. While these assays have revealed novel insights into HIV-1 proviral landscape dynamics, some of the techniques are expensive, labor-intensive, and require sophisticated equipment, making them unsuitable for use in large-scale clinical trials, especially in resource-limited settings where the HIV-1 pandemic is more prevalent. In addition, these assays exhibit high assay fail rates due to HIV-1 sequence polymorphisms within the primer-probe binding regions and near-full length genome sequencing methods may introduce a quantification bias. Importantly, proviral DNA sequence intactness alone is not a direct measure of inducibility, which greatly depends on the site of viral integration in the host genome and therein the potential for viral transcription.

On the opposite end of the spectrum of reservoir quantitation, the culture-based, quantitative viral outgrowth assay (QVOA) is considered a gold standard technique to detect the outgrowth of replication-competent virus and therefore measures the functional viral reservoir. However, QVOA underestimates the size of the viral reservoir due to suboptimal induction and/or inadequate propagation of all replication-competent viruses *in vitro;*. Moreover, QVOA requires a high input of CD4+ T cells that necessitate

large volume blood draws (>100 mL), is labor-intensive and time-consuming (14 to 21 days), limiting its use in routine clinical settings.

Novel culture-based techniques have emerged as potential alternate assays to estimate the size of the inducible viral reservoir. These methods involve the activation of resting or total CD4+ T cells from virally suppressed individuals, followed by the direct measurement of HIV-1 RNA from cell extracts (cell-associated RNA, ca-RNA) or cell culture supernatants (cell-free RNA, cf-RNA). However, it should be noted that assays relying on quantification of cf-RNA may not accurately assess replication-competency as it only reflects the capacity to generate and release viral RNA. Inducible ca-RNA assays, on the other hand, involve the potent activation of CD4+ T cells by anti-CD3/CD28 antibodies, phorbol 12-myristate 13-acetate (PMA) and ionomycin, or latency reversing agents (LRAs), followed by an ultrasensitive PCR specific for a given viral transcript. Different RNA species including unspliced RNA (us RNA), multiply spliced RNA (msRNA), mature RNA transcripts with poly-A tails, TAR RNAs, and chimeric host-HIV-1 read-through transcripts can be quantified by RT-qPCR or RT-ddPCR methods. Tat/Rev msRNA in particular has been found to be a meaningful indicator of viral replication following latency reversal since Tat/Rev msRNA transcripts are generated after splicing of full-length viral transcripts. By detecting Tat/Rev msRNA therefore, the likelihood that proviruses with large internal deletions are measured is greatly reduced. Inducible HIV-1 RNA assays can be conducted in a limiting dilution format, such as the Tat/Rev Limiting Dilution Assay (TILDA), or at single-cell level by, for example, detection of HIV-1 usRNA and/or msRNA using fluorescent in situ hybridization assays coupled to flow cytometry (Fish-flow) which provide a deeper understanding of the viral reservoir molecular characteristics and phenotypic heterogeneity. However, these approaches still require high starting input (>50 mL of blood) and take 3 days to perform, which make FISH-flow assays less suitable for use in large scale cure intervention trials. Therefore, in most clinical studies viral RNA

transcripts are preferably measured in bulk or in serial-diluted samples using quantitative real time or digital PCR-based methods.

HIV-1 RNA quantification is typically performed using single round or semi-nested quantitative reverse transcription real-time PCR (RT-qPCR). Semi-nested RT-qPCR is especially useful for assessing samples with low copies of the target RNA and offers a wider dynamic range compared to single-round qPCR assays. However, when HIV-1 RNA is quantified in limiting dilution format, e.g., in 96 wells or 384 wells, the cost of reagents considerably increases. Moreover, the instrument-intensive amplification procedure, long turnaround time, and high risk of cross-contamination from manual handling of pre-amplified product in semi-nested RT-qPCR protocols, limits assay application in large clinical trials, especially in resource-constrained settings.

To address these technical limitations, reverse transcription loop-mediated isothermal amplification (RT-LAMP) could be used as a highly sensitive, less expensive and time-saving alternative to semi-nested RT-qPCR. RT-LAMP uses two different polymerase enzymes to promote reverse transcription and isothermal amplification in a single reaction, which significantly reduces the risk of cross-contamination. RT-LAMP has been successfully used in multiple studies to detect low copies of viral RNA for SARS-CoV-2 diagnosis, demonstrating comparable or better sensitivity than conventional RT-qPCR. Additionally, by targeting six to eight different DNA regions, RT-LAMP is more specific than semi-nested PCR, which targets three regions. Although RT-LAMP has the potential to be a less expensive and robust alternative to (semi-nested) RT-qPCR or RT-ddPCR, it has, thus far, only been used for qualitative HIV-1 RNA detection in blood plasma and not yet in the context of HIV-1 reservoir quantification. Here, the present inventors present SQuHIVLa (Specific Quantification of Inducible HIV-1 reservoir by LAMP), a new HIV-1 reservoir assay that leverages RT-LAMP to detect HIV-1-infected cells that spontaneously or inducibly express Tat/Rev msRNA.

The present invention provides a method for determining whether a sample comprises inducible HIV-1, comprising performing a reverse transcription, loop-mediated isothermal amplification (RT-LAMP) reaction with said sample with a primer set specific for Tat/Rev multiply spliced HIV-1 RNA and determining whether the sample comprises an amplification product of the amplification reaction.

In one embodiment of a method of the invention, the binding sites for the F2 and F1 LAMP primers on the amplification template are selected such that the hairpin loop between and including the F2 and F1 primers, that forms as a result of primer extension from the 'F3' primer and the subsequent displacement of the forward inner primer product, spans a region in the Tat/Rev ms HIV-1 RNA (or the cDNA template thereof obtained after reverse transcription) that overlaps with the splice site of the intron interrupting the Tat and Rev coding sequences. The term "a region that overlaps with the splice site" means that primer extension from these primers will also occur when annealing to the unspliced RNA or DNA form of Tat/Rev HIV-1 RNA. However, when the unspliced RNA or DNA form of Tat/Rev HIV-1 RNA is the template, the hairpin loop between and including the F2 and F1 primers will include the intron that is about 2 kbp in size, resulting in an unstable loop in the reaction product comprising the F2-intron-F1 sequence, which loop will therefore not be efficiently amplified and will not result in an amplification product.

In an alternative embodiment of a method of the invention, the binding sites for the B2c and B1c LAMP primers on the amplification template are selected such that the hairpin loop between and including the B2c and B1c primers, that forms as a result of primer extension from the B3c primer and the subsequent displacement of the backward inner primer product, spans a region in the Tat/Rev ms HIV-1 RNA (or the cDNA template thereof obtained after reverse transcription) that overlaps with the splice site of the intron interrupting the Tat and Rev coding sequences.

Similarly, when the unspliced RNA or DNA form of Tat/Rev HIV-1 RNA is the template, the hairpin loop between and including the B2c and B1c primers will include the intron that is about 2 kbp in size, resulting in an unstable loop in the reaction product comprising the B2c-intron-B1c sequence, which loop will therefore not be efficiently amplified and will not result in an amplification product.

One of skill in the art will be able to perform the method of the invention, and will appreciate that primer sets for LAMP amplification can be generated using computer programs that are available online, such as the PrimerExplorer V5 online software.

In methods of this invention, the LAMP reaction for detection of HIV-1 is subtype specific. However, it was found that the genome organization of the various HIV-1 subtypes allows for the method to be standardized, in that the preferred primer binding sites for the F2 and B2c primers are located in the region defined by nucleotide positions 55 to 375 of the sequence of said Tat/Rev ms HIV-1 RNA.

With respect to the selection of the template binding site for the F2 and F1 primers or the template binding site for the B2c and B1c primers, it was determined that the Tat/Rev intron splice site is located as position 215 (+/- 1 or 2 nucleotides) in the Tat/Rev ms RNA sequence. To ensures unstable loop formation, while the primers are bound to intron-containing Tat/Rev DNA, and inhibition of isothermal amplification, primer sets that possess the F2 binding region within the splice site and 150 nucleotides upstream of the splice site (approximately, nt pos. 65 to 215 of the Tat/Rev ms RNA sequence) and B2c binding region within 25 nucleotides upstream of the splice site and 150 nucleotides downstream of the splice site (approximately, nt pos. 190 to 365 of the Tat/Rev ms RNA sequence) are suitable for use in the present invention.

Therefore, when reviewing any LAMP primer set, such as for instance generated by computer, the primer binding sites for the F2 and B2c

primers are in one embodiment preferably located in the region defined by nucleotide positions 55 to 375 of the sequence of the Tat/Rev ms HIV-1 RNA.

Furthermore, in one preferred embodiment, wherein the Tat/Rev splice site falls within forward loop 5'-F2 to F1-3', the primer binding site for the F2 primer may be located in the region defined by nucleotide positions 125 to 240 of the sequence of the Tat/Rev ms HIV-1 RNA. In another preferred embodiment, wherein the Tat/Rev splice site falls within backward loop 5'-B2c to B1c-3', the primer binding site for the B2c primer may be located in the region defined by nucleotide position 190 to 305 of the sequence of the Tat/Rev ms HIV-1 RNA. Preferably, the primer binding sites for the B2c and B1c primers is located in the region defined by nucleotide positions 190 to 365 of the sequence of the Tat/Rev ms HIV-1 RNA.

The distance from the 5' end of F2 to the 3' end of B2c is preferably about 200 bases, more preferably between about 120 to 160 bases. The distance from the 5' end of F2 to the 5' end of F1, which region forms the loop, is preferably between about 40 and 60 bases. Additionally, the distance between F2 and F3 is preferably between 0 and 60 bases to optimize the amplification process.

Furthermore, with respect to the selection of the template binding site for the F2 and F1 primers, it is preferred that these positions are selected such that between the 3' end of the F2 primer and the 5' end of the F1 primer, a region of at least 22 nucleotides is reserved for the inclusion of a forward loop primer (FL), and/or that between the 5' end of the B2c primer and the 3' end of the B1c primer, a region of at least 22 nucleotides is reserved for the inclusion of a backward loop primer (FL).

It is further preferred that these loop primers can be converted into a self-quenching probe as described in Gadkar *et al.*, 2018 (Scientific Reports 8, 5548) or molecular beacon as described in Tyagi *et al.*, 1996 (Nat. Biotechnol. 14, 303–308). To convert one of the loop primers into a self-quenching probe or molecular beacon as described herein, the sequence of the loop primer forms the target-specific sequence of the self-quenching

probe or molecular beacon. In such embodiments, the target-specific sequence is complementary to the target sequence of the amplicon.

One of skill in the art will appreciate the following general features that may be taken into account for LAMP primer selection. With respect to the melting temperature (Tm, °C) of the primers, these may be estimated using the Nearest-Neighbor method, which is considered the most accurate approximation method. Precise determination of Tm for each LAMP primer set may support proper initiation of DNA synthesis from LAMP inner primers and the timely formation of loop structures following the release of single-stranded DNA from the template. With respect to the GC content of the primers, this may typically fall within the range of 40% to 65%. It should be noted, however, that primers with a GC content lower than 40% may be necessary in cases where the DNA template is particularly AT-rich. With respect to secondary structure, primers should preferably not form secondary structures, notably the LAMP inner primers. Formation of primer dimers is best avoided by avoiding 3' ends complementarity between the primers.

In order to provide suitable LAMP primer sets for use in aspects of this invention, one of skill in the art will appreciate that a consensus sequence of *in silico* spliced Tat/Rev ms HIV-1 RNA sequences of a plurality of distinct HIV-1 isolates of a single HIV-1 subtype may for instance be produced by selecting reference HIV-1 genome sequences from public databases. Preferably, for a specific subtype of HIV-1, a minimum of 10 complete genome sequences of the desired subtype may be downloaded from the Los Alamos database using the sequence search interface webpage (https://www.hiv.lanl.gov/components/sequence/HIV/search/search.html). The desired subtype and the complete genome option may be selected for searching. The sequences submitted from different geographic locations (such as African, Asian, European countries etc.) and in different years (for instance, sequences submitted before 2000, between 2000-2010 and 2010-present) may be chosen to account for sequence diversity and to increase the

probability that the primers recognize and bind to Tat/Rev msRNA from a majority of distinct HIV-1 isolates of a single HIV-1 subtype. The selected sequences may for instance be downloaded and saved in FASTA file format.

In order to provide a consensus sequence for the plurality of distinct HIV-1 isolates of a single HIV-1 subtype, a variety of different software options are available for sequence alignment, both online and as stand-alone applications. Use may be made of Molecular Evolutionary Genetics Analysis (MEGA) software for this purpose (Sudhir Kumar, Koichiro Tamura, and Masatoshi Nei. 1993. MEGA: Molecular Evolutionary Genetics Analysis, version 1.01. The Pennsylvania State University, University Park, PA 16802, and updates of that program), for instance using the built-in MUSCLE algorithm to align the downloaded reference genome sequences.

The resulting alignment is used to manually curate the Tat/Rev sub-genomic region, approximately 480 nucleotides, excluding the intron region (approximately nucleotide position 6046 to 8378, relative to the coordinates of 5' LTR U3 start genome) (Los Alamos HIV-1 sequence compendium, 2021). The consensus sequence of the curated Tat/rev region is downloaded and saved in FASTA file format.

The above referred consensus sequence may be used in aspects of this invention. In preferred embodiments, a consensus sequence as set forth in any one of Figures 10-15 and sequences having at least 75%, at least 80%, at least 85%, at least 90%, at least 95% and more preferably at least 98% sequence identity thereto are used in aspects of this invention. These consensus sequences are HIV-1 subtype specific.

Figures 10-15 provide exemplary consensus sequences for the multiply spliced version of the Tat/Rev sequence of various HIV-1 subtypes. These sequences do not contain introns. The consensus sequence is formed by the combination of the sequence of Tat exon 1 followed by Rev exon 2 (i.e., excluding the intron). GenBank accession AF033819.3, provides a complete reference sequence for HIV-1 subtype B. The location of the

consensus sequence in the Genbank reference sequence AF033819.3 (Apr 2, 1999) of the HIV-1 genome runs from position 5377 to 5591 for the first 215 nucleotides (first exon of Tat), and continues from position 7925 to 8199 for the remaining nucleotides (second exon of Rev). The nucleotide position 55-375 as used herein refers to the numbering in the consensus sequences. And the consensus sequence is generated by combining sequence of Tat exon 1 followed by Rev exon 2 (excluding the intron).

Following the provision of a HIV-1 subtype-specific Tat/Rev ms HIV-1 RNA consensus sequence, PrimerExplorer V5 online software may be used to provide a preliminary primer set for LAMP amplificaton. As the input target sequence file, the Tat/Rev DNA FASTA sequence (about 480 bp), representing a subtype-specific consensus sequence, may be used. Program parameters may be modified as desired from the default settings prior to executing the primer design algorithm. Preferably, for instance, the sequence parameter condition may be set to "AT rich" as the GC content of the input sequences may vary between 35-45% depending on the subtype. In addition, the Tm for F1c/B1c was set to 60°C-64°C, while the Tm for F3/B3 and F2/B2 was set to 55°C-60°C to ensure the generation of a sufficient number of potential LAMP primer sets. The software then displays a maximum of about 1000 potential primer sets using a combination of different potential primer binding sites.

From the potential primer sets generated by the software, the primer prerequisites as described herein for binding position with respect to the Tat/Rev intron splice site, mutual distance, melting temperature, etc., are included when selecting a suitable primer set from the potential primer sets generated by the software. After selecting a primer set that fulfills the requirements for exclusive binding of msRNA, and suitable for designing the loop primers, primer binding sites belonging to different primer sets may be combined to form a custom set as long as they have a similar Tm for F3/B3, F2/B2, and F1c/B1c and fulfill the specific distance requirement between LAMP primer binding regions.

After selecting a suitable primer set, the sequences and primer information files may be downloaded from the software and utilized for designing the Loop primers (LF/LB). Hereto, the primer information of the selected suitable primer set is used as input to generate potential loop primer sequences, for instance by using the PrimerExplorer V5 software. However, since the sequence of one of the loop primers is preferably used as the target-specific sequence of a self-quenching and internal FAM fluorophore-containing probe (or molecular beacon), the presence of a cytosine (C) or guanine (G) residue at the terminal 3′ end of the loop primer is preferred. Further preferred is a T residue at the second or third position from this 3′ end, and as an optional preferred embodiment, the primer comprises one or more G nucleotides flanking the T residue (optional).

In case no suitable loop primers can be selected from the software-generated list, the closest possible sequence is chosen, and manual modifications are performed to ensure its applicability as a probe. Hereto, the primer binding regions of the suitable primer set may be aligned with the original reference HIV-1 sequences for providing the consensus sequence as described above. Mutation hotspots located within primer binding regions that would result in primer-template mismatches may then be identified. Based thereon, selected suitable primers may be moved upstream or downstream of the envisioned primer binding site to ensure that the primers contain conserved nucleotides at specific positions that are important for amplification to proceed (3' end of F2/B2 and F3/B3, and 5' end of F1c/B1c). Mutation hotspots may be permitted at less essential regions (5' end of F2/B2 and F3/B3, 5' end of the F1c/B1c, and the internal region) where a mismatch will have less effect on amplification. Tm may then be recalculated for each modified primer to ensure that the new primers have similar Tm to the original primers, and the distance between the modified primer regions must also meet specific requirements to be considered as LAMP primers. Distances between the primer binding regions may be calculated from the alignment and the Tm of the modified primers

may be calculated using Kun's Oligonucleotide Tm calculator (https://arep.med.harvard.edu/kzhang/cgi-bin/myOligoTm.cgi). The calculated Tm may be affected by experimental conditions such as the salt concentration and oligonucleotide concentration. It is therefore preferred that Tm be calculated under preferred experimental conditions (oligonucleotide concentration preferably at 0.1 μM, sodium ion concentration preferably at 50 mM, magnesium ion concentration preferably at 4 mM).

Similarly, selected loop primers are aligned with the reference HIV-1 complete genome sequences and modified following the same procedures (moving upstream or downstream of the current primer binding site). This ensures that one of the loop primers fulfills the requirements to be converted into a self-quenching probe and that the internally labeled T and 3' end G/C of the probe region are conserved among the HIV-1 sequences used for the alignment.

A method of the invention comprises determining whether the sample comprises an amplification product of the amplification reaction. In preferred embodiments this comprises detecting the presence or absence of amplification product. Such detection can be performed with any method known in the art for detection of amplification product of a LAMP reaction, including but not limited to the use of fluorescent intercalating dyes, fluorescent primers or probes, measuring turbidity, electrochemical probes, bioluminescent signals, chemiluminescent probes, changes in pH and magnesium based dyes.

In some embodiments detecting the presence or absence of amplification product comprises detecting the presence or absence of binding of a double-stranded DNA binding dye or intercalating dye into the amplification product. A method of the invention may therefore comprise a dye-binding step. A dye-binding step generally includes contacting the amplification products with a double-stranded DNA binding or intercalating dye. The presence of binding is typically indicative of the presence of

Tat/Rev msRNA amplification product and thus of inducible HIV-1 in the sample. Hence, amplification product is detected if binding is detected. The absence of binding is typically indicative of the absence of Tat/Rev msRNA amplification product and thus of inducible HIV-1 in the sample. Hence, amplification product is not detected if binding is detected. A method of the invention may further include the step of determining the melting temperature between the amplification product and the double-stranded DNA binding dye. Generally, the melting temperature confirms the presence or absence of Tat/Rev msRNA amplification product and thus of inducible HIV-1 in the sample. Suitable double-stranded DNA binding or intercalating dyes include SYTO-9®, SYTO-13®, SYTO-82®, SYBR Green I®, SYBR Gold® and EvaGreen®.

In some embodiments detecting the presence or absence of amplification product comprises measuring turbidity in the reaction mixture during the amplification reaction. Turbidity measurement is based on turbidity derived from magnesium pyrophosphate formation in the reaction mixture. The LAMP method allows to synthesize large amounts of DNA. Consequently, a large amount of pyrophosphate ion, a by-product of DNA synthesis, is produced. This yields white precipitate of magnesium pyrophosphate in the reaction mixture. Measuring the presence or absence of this precipitate allows detecting the presence or absence of amplified nucleic acid by the LAMP reaction. And because an increase in the turbidity of the reaction mixture according to the production of precipitate correlates with the amount of DNA amplified, real-time monitoring of the LAMP reaction is possible by real-time measurement of turbidity.

In some preferred embodiments, the step of determining whether the sample comprises an amplification product comprises monitoring amplification during the amplification process, i.e. real-time detection and/or monitoring of the amplification reaction. Real-time detection and monitoring is possible for both detection based on double-stranded DNA binding or intercalating dyes or on turbidity measurements.

The present inventors demonstrate that reverse transcription loop-mediated isothermal amplification (RT-LAMP) can be used for the rapid, specific and sensitive detection of Tat/Rev ms HIV-1 RNA. The RT-LAMP reaction of the present invention specifically amplifies Tat/Rev ms HIV-1 RNA and not intron-containing Tat/Rev genomic DNA. RT-LAMP utilizes two different polymerase enzymes to promote reverse transcription and isothermal amplification in a single reaction, which significantly reduces the risk of cross-contamination. The use of RT-LAMP for the detection of Tat/Rev ms HIV-1 RNA is demonstrated herein, and shows comparable or better sensitivity than conventional RT-qPCR. RT-LAMP for the detection of Tat/Rev ms HIV-1 RNA may target six to eight different regions for primer annealing versus three in semi-nested PCR. Hence, RT-LAMP is more specific, and has the potential to be a less expensive and robust alternative to (semi-nested) RT-qPCR or RT-ddPCR. The present inventors demonstrate its use in HIV-1 reservoir quantification.

The methods of the present invention, in some embodiments, comprise RT-LAMP-based detection of Tat/Rev msRNA from activated HIV-1-infected $CD4^+$ T cells distributed in a limiting dilution format as shown in the Examples (4 and 5). In these Examples, which are to be understood as non-limiting to the present invention, but merely as an illustration, $CD4^+$ T cells obtained from virally suppressed people with HIV-1 (PLWH), were potently stimulated with PMA and ionomycin, to induce high levels of viral transcription. Following stimulation, serial dilutions of cells were prepared for RT-LAMP amplification of Tat/Rev ms HIV-1 RNA transcripts whereby the amplification was performed directly on whole cells. The frequency of cells expressing Tat/Rev ms HIV-1 RNA was then determined using the maximum likelihood method, but it will be understood that other statistical methods for estimating the frequency of infected cells containing inducible replication-competent HIV-1 may also be employed.

The invention further provides a kit of parts comprising a LAMP primer set according to the invention and a DNA polymerase, preferably an

RT-LAMP primer set according to the invention including a reverse transcriptase and a DNA polymerase, set kit of parts further comprising nucleotides (for instance as commercially available in the form of a dNTP mixture), and/or a reaction buffer.

In preferred embodiments, the DNA polymerase in a kit of parts of the invention is a polymerase from *Bacillus stearothermophilus* (Bst) or a homologue thereof, also referred to as a Bst DNA polymerase, such as Bst long fragment (Bst-Lf), a Bst2.0 DNA polymerase, a Bst3.0 DNA polymerase or DNA polymerase I of *Parageobacillus yumthangensis* or a homologue thereof, such as the long fragment thereof. In further preferred embodiments, reaction buffer present in a kit of parts of the invention comprises one or more components selected from guanidine, ammonium sulphate, tween, preferably at least two of said components, preferably all three. It is further preferred that at least guanidine and ammonium sulphate are present in the reaction buffer. If present, guanidine is preferably present in the reaction buffer in a concentration of 20-100 mM, more preferably from 25-60 mM, more preferably from 30-50 mM, e.g. 35-45 mM or about 40 mM. If present, ammonium sulphate is preferably present in the reaction buffer in a concentration of 15-100 mM, more preferably from 20-50 mM, more preferably from 20-40 mM, e.g. 25-30 mM. If present, tween is preferably present in the reaction buffer in a concentration of 0.05-1 wt% of Tween20, or a corresponding concentration of another tween product, more preferably 0.05-0.5 % v/v, more preferably 0.06 – 0.2 % v/v, e.g. 0.075-0.125 % v/v or about 0.1 % v/v. As used herein, a corresponding concentration of another tween product refers to a concentration for the product that provides the same properties as the indicated concentration of Tween20. In particularly preferred embodiments, the DNA polymerase is a Bst polymerase or a homologue thereof, such as Bst long fragment (Bst-Lf), a Bst2.0 DNA polymerase, a Bst3.0 DNA polymerase or DNA polymerase I of *Parageobacillus yumthangensis* or a homologue thereof, such as the long fragment thereof, and the reaction buffer comprises one or more components

selected from guanidine, ammonium sulphate, tween, preferably in the concentrations indicated above, preferably the reaction comprises at least two of said components, preferably all three, more preferably at least guanidine and ammonium sulphate, preferably in the concentrations indicated above.

The kit of parts of the invention may comprise a reverse transcriptase for performing a RT-LAMP reaction.

The kit of parts of the invention may comprises one or more containers, each container comprising the primer set of the invention, or a primer of the primer set, the DNA polymerase, the nucleotides and/or the reaction buffer.

The kit of parts of the invention may also include instructions for use, in particular for using the primers, reverse transcriptase and DNA polymerase, nucleotides and/or reaction buffer to amplify and detect the presence or absence of Tat/Rev ms HIV-1 RNA or inducible HIV-1 in a sample.

In preferred embodiments, the kit of the invention comprises a LAMP primer set comprising a forward outer primer (F3), a backward outer primer (B3), a forward inner primer (FIP) and a backward inner primer (BIP), and optionally further comprising a loop forward primer (LF) and/or loop backward primer (LB), preferably both a loop forward and loop backward primer, preferably one of said primers is labeled with a detectable label as described herein.

In some embodiments, the kit comprises a primer set specific for Tat/Rev ms HIV-1 RNA as defined herein.

The kit may comprise a primer set specific for Tat/Rev ms HIV-1 RNA of any HIV-1 subtype. For instance, the primer set may be specific for Tat/Rev msRNA of HIV-1 subtype B, subtype C, subtype A, subtype D, subtype AE or subtype AG. Preferably, the primer set may be specific for RT-LAMP amplification of Tat/Rev msRNA of HIV-1 of one of the consensus sequences of Figures 10-15.

In preferred embodiments, for amplifying Tat/Rev ms HIV-1 DNA from HIV-1 subtype B:

primer F3 comprises a consecutive stretch of at least 15 nucleotides of the sequence GTGTTGCTTTCATTGCCAAG, more preferably 15, 16, 17, 18, 19, or 20 nucleotides of said sequence, more preferably 18, 19, or 20 nucleotides of said sequence, more preferably 19 or 20 nucleotides of said sequence;

primer B3 comprises a consecutive stretch of at least 15 nucleotides of the sequence GTCTCTCTCTCCACCTTCT, more preferably 15, 16, 17, 18 or 19 nucleotides of said sequence, more preferably 17, 18 or 19 nucleotides of said sequence, more preferably18 or 19 nucleotides of said sequence;

primer FIP comprises a consecutive stretch of at least 30 nucleotides or two consecutive stretches of at least 30 nucleotides in total of the sequence TGAGGAGCTCTTCGTCGCTGCAAAAGCCTTAGGCATCTC, more preferably 33, 34, 35, 36, 37, 38, or 39 nucleotides of said sequence, more preferably 37, 38, or 39 nucleotides of said sequence;

primer BIP comprises a consecutive stretch of at least 30 nucleotides or two consecutive stretches of at least 30 nucleotides in total of the sequence CAGTCAGACTCATCAAGTTTCTCTCTTCGATTCCTTCGGGCC, more preferably 35, 36, 37, 38, 39, 40, 41, or 42 nucleotides of said sequence, more preferably 39, 40, 41, or 42 nucleotides of said sequence, more preferably 41 or 42 nucleotides of said sequence;

primer LF comprises a consecutive stretch of at least 13 nucleotides of the sequence TCTCCGCTTCTTCCTGC, more preferably 14, 15, 16, or 17 nucleotides of said sequence, more preferably 15, 16 or 17 nucleotides of said sequence, more preferably 16 or 17 nucleotides of said sequence, and

primer LB comprises a consecutive stretch of at least 13 nucleotides of the sequence AACCCACCTCCCAACCC, more preferably 14,

15, 16, or 17 nucleotides of said sequence, more preferably 15, 16 or 17 nucleotides of said sequence, more preferably 16 or 17 nucleotides of said sequence.

In preferred embodiments, for amplifying Tat/Rev ms HIV-1 DNA from HIV-1 subtype C:

primer F3 comprises a consecutive stretch of at least 15 nucleotides of the sequence TCCTTGTAATAAGTGTTATTGTAA, more preferably 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides of said sequence, more preferably 22, 23, or 24 nucleotides of said sequence, more preferably 23 or 24 nucleotides of said sequence;

primer B3 comprises a consecutive stretch of at least 15 nucleotides of the sequence CGATTCTTCCGAGCCTGTC, more preferably 15, 16, 17, 18 or 19 nucleotides of said sequence, more preferably 17, 18 or 19 nucleotides of said sequence, more preferably18 or 19 nucleotides of said sequence;

primer FIP comprises a consecutive stretch of at least 30 nucleotides or two consecutive stretches of at least 30 nucleotides in total of the sequence CCGCTTCTTCCTGCCATAGGAATAGCTATCATTGTCTAGTTTGC, more preferably 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, or 44 nucleotides of said sequence, more preferably 41, 42, 43, or 44 nucleotides of said sequence, more preferably 43 or 44 nucleotides of said sequence;

primer BIP comprises a consecutive stretch of at least 30 nucleotides or two consecutive stretches of at least 30 nucleotides in total of the sequence CGACGAAGCGCTCCTCCAAGGTTTGGGGTAAGGGTTGCT, more preferably 33, 34, 35, 36, 37, 38, or 39 nucleotides of said sequence, more preferably 36, 37, 38, or 39 nucleotides of said sequence, more preferably 38 or 39 nucleotides of said sequence;

primer LF comprises a consecutive stretch of at least 13 nucleotides of the sequence TGCCTAAGCCTTTTGTCTG, more preferably 14, 15, 16, 17, 18, or 19 nucleotides of said sequence, more preferably 17, 18,

or 19 nucleotides of said sequence, more preferably 18 or 19 nucleotides of said sequence, and

primer LB comprises a consecutive stretch of at least 13 nucleotides of the sequence CAGTGAGGATCATCAAAATC, more preferably 14, 15, 16, 17, 18, 19, or 20 nucleotides of said sequence, more preferably 18, 19, or 20 nucleotides of said sequence, more preferably 19 or 20 nucleotides of said sequence.

In some preferred embodiments, the kit of parts comprises a primer set specific for more than one HIV-1 subtype, such as a primer set for two or three HIV-1 subtypes selected from subtype B, subtype C, subtype A, subtype D, subtype AE or subtype AG according to the invention.

The invention further provides a use of a primer set or primer sets or kit of parts according to the invention for determining whether a sample comprises inducible HIV-1, in particular using a method according to the invention.

The invention further provides a use of a primer set or primer sets or kit of parts according to the invention for the detection of inducible HIV-1 in a subject receiving or having received antiretroviral therapy (ART), or for quantifying the inducible HIV-1 reservoir in such a subject, in particular using a method according to the invention.

The invention further provides a use of a primer set or primer sets or kit of parts according to the invention for detecting Tat/Rev multiply spliced HIV-1 RNA in a sample.

The invention further provides a use of a primer set or primer sets or kit of parts according to the invention for detecting an amplification product of Tat/Rev multiply spliced HIV-1 RNA in a sample.

Features may be described herein as part of the same or separate aspects or embodiments of the present invention for the purpose of clarity and a concise description. It will be appreciated by the skilled person that the scope of the invention may include embodiments having combinations of

all or some of the features described herein as part of the same or separate embodiments.

The invention will be explained in more detail in the following, non-limiting examples.

EXAMPLES

Methods

Cohort characteristics

Peripheral blood samples from 25 individuals with HIV-1 (6 subtype B and 19 subtype C) were used in this study. All the participants were older than 18 years of age and on fully suppressive combination antiretroviral therapy (cART), initiated during chronic stages of HIV-1 infection, with plasma HIV-1 RNA below detectable levels (< 20 copies/mL) for at least twelve months.

Sample collection

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood or leukapheresis samples using Ficoll density gradient centrifugation and cryopreserved in liquid nitrogen until further use. CD4+ T cells were isolated from thawed PBMCs by negative magnetic selection using the EasySep Human CD4 T Cell Enrichment Kit (STEMCELL Technologies) according to the manufacturer's protocol.

Designing of Tat/Rev RNA-specific LAMP primer/probe sets

Briefly, ten complete HIV-1 genome sequences of a particular subtype, submitted from various geographical locations and from different years, were obtained from the Los Alamos HIV sequence databases (https://www.hiv.lanl.gov/) (GenBank Accession numbers of the sequences

used to design Subtype B and C Tat/Rev msRNA LAMP primers are listed in Table 1).

Table 1: GenBank accession numbers of the sequences used to design Tat/Rev HIV-1 msRNA specific LAMP primers and probes.

GenBank Accession numbers	**Subtype**	**Location**	**Sampling year**
AF033819.3	B	USA	2018
A07867	B	France	1983
AB221126	B	Japan	2004
AB287364	B	Japan	2005
AB287367	B	Japan	2005
AB485638	B	USA	1991
AF042102	B	Australia	1993
AY423384	B	Netherlands	2000
AY682547	B	Russia	2004
AY779552	B	Canada	2000
AF067158	C	India	1993
AF110975	C	Botswana	1996
AY463220	C	South Africa	2000
AB254143	C	Zambia	2002
DQ369977	C	South Africa	2003
KX907339	C	Tanzania	2003
JX976688	C	South Africa	2005
MT194744	C	Zambia	2006
KU319541	C	Ethiopia	2008
KY112200	C	Malawi	2008

The sequences were aligned using the Molecular Evolutionary Genetics Analysis software version 11 (MEGA11; Tamura, K., Stecher, G. & Kumar, S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution 38, 3022-3027 (2021)) and manually modified to generate a new alignment of *in silico*-spliced Tat/Rev DNA sequences (approximately 490 bp). The consensus Tat/Rev DNA sequence of this second alignment was then converted to a FASTA file and used to generate preliminary sets of LAMP primers using the online program PrimerExplorer V5 (world wide web (WWW) address: primerexplorer.jp/e/).

Selected LAMP primers, based on criteria that we have identified to be crucial to ensure precise detection of Tat/Rev msRNA transcripts, were aligned to the complete HIV-1 genome alignment to assess primer-target sequence complementarity. To broaden the detection of multiple strains within a particular HIV-1 subtype, the primer binding regions with mutations present at sites extremely important for amplification were avoided by shifting these regions several nucleotides up or downstream. Importantly, the melting temperature of the new primers and the distance between the primer binding regions were re-calculated to ensure that the modifications still met the initial LAMP primer specifications. Additionally, loop primers were generated using the PrimerExplorer V5 software and one of these (e.g., Loop forward primer) was converted into a self-quenching probe with an internal FAM fluorophore bound to the closest thymidine from the 3' end (Gadkar, V.J., Goldfarb, D.M., Gantt, S. & Tilley, P.A.G. Real-time Detection and Monitoring of Loop Mediated Amplification (LAMP) Reaction Using Self-quenching and De-quenching Fluorogenic Probes. Scientific Reports 8, 5548 (2018).). A more detailed protocol for designing these HIV-1 Tat/Rev msRNA LAMP primers and probe is provided below:

Steps to design HIV-1 Tat/Rev msRNA LAMP primers:

1) Select reference HIV-1 genome sequences

To design LAMP primers for a specific subtype of HIV-1, download a minimum of 10 complete genome sequences of the desired subtype from the Los Alamos database using the sequence search interface webpage (https://www.hiv.lanl.gov/components/sequence/HIV/search/search.html). Select sequences submitted from different geographic locations (such as, Africa, Asia, Europe and USA) and in different years (e.g., sequences submitted before 2000, between 2000-2010 and 2010-present). This selection is necessary to account for sequence diversity and increase the probability

that the designed primers will recognize and bind to Tat/Rev msRNA from a majority of individuals with the selected HIV-1 subtype. Download the selected reference HIV-1 genome sequences, save in FASTA file format.

2) Generate a spliced Tat/Rev consensus sequence in silico

Align the downloaded reference HIV-1 genome sequences using a sequence alignment tool of choice. We utilized the built-in MUSCLE algorithm in MEGA11 software. Manually edit the aligned sequences by excluding the following sequence regions; upstream of the Tat gene (1 to 5830); Tat/rev intron (6046 to 8383) and downstream of the Rev gene(8654 to 3'end). The nucleotide positions are relative to the coordinates of the 5' LTR UR start of the reference HIV-1 genome (ID) provided in the Los Alamos HIV-1 sequence compendium (2021). The resulting alignment of the spliced Tat/Rev sub-genomic region should be approximately 490 nucleotides. Download the spliced Tat/Rev DNA consensus sequence, save in FASTA file format.

Generate a preliminary LAMP primer set using the PrimerExplorer V5 software

Upload the Tat/Rev DNA consensus FASTA file (i.e., the target sequence file) into the online PrimerExplorer V5 software. Adjust the default settings prior to executing the primer design algorithm. Set the sequence parameter condition to "AT rich" ; the GC content of the Tat/rev sequence varies from 35-45% depending on the HIV-1 subtype. Set the Tm for F1c/B1c to 60°C-64°C, and set the Tm for F3/B3 and F2/B2 to 55°C-60°C to ensure the generation of a sufficient number of potential LAMP primer sets. The software displays a maximum of 1000 primer sets using a combination of different primer binding sites.

Several prerequisites need to be considered when selecting the preliminary primer set from the potential LAMP primer sets generated by the software.

- To ensure the specificity of the primer set for Tat/Rev msRNA and not the intron-containing Tat/Rev DNA, only primer sets containing the splice site of msRNA (approximately, the 215thnucleotide) within the F2 to F1c or B2 to B1c primer binding regions should be considered. This ensures that unstable loop formation, while the primers are bound to intron-containing Tat/Rev DNA, would result in the inhibition of isothermal amplification. Therefore, select potential primer sets where the F2 binding region is within 25 nucleotide downstream of the splice site and 90 nucleotides upstream of the splice site (approximately, nt pos. 125 to 240) or where the B2 binding region is within 25 nucleotides upstream of the splice site and 90 nucleotides downstream of the splice site (approximately, nt pos. 190 to 305).
- Since loop primers, which accelerate the LAMP reaction, are also used for amplification, only primer sets that have a distance of >22 nucleotides between F2 and F1c/B2 and B1c should be considered for the preliminary primer set.
- If impossible to find one primer set that fulfills both requirements for exclusive binding of msRNA, and suitable for designing the loop primers, then primer binding sites belonging to different primer sets could be mixed to form a custom set as long as they have a similar Tm for F3/B3, F2/B2, and F1c/B1c and fulfill the specific distance requirement between LAMP primer binding regions.

After selecting the preliminary primer set, download the sequences and primer information files from the software to use for designing the Loop primers (LF/LB).

3) Design loop primers

Upload the LAMP primer information files into the PrimerExplorer V5 software. Since one of the loop primers needs to be converted into a self-quenching and internal FAM fluorophore-containing probe, specific criteria should be considered when selecting the loop primers:

- presence of a cytosine (C) or guanine (G) residue at the terminal 3′ end
- a thymine (T) residue at the second or third position from this 3′ end
- one or more G nucleotides flanking the T residue (optional).

In case no suitable loop primers can be selected from the software-generated list, the closest possible sequence is chosen, and manual modifications are performed to ensure its applicability as a probe (detailed below).

4) Adapt LAMP primer and probe sequences

The Tat/Rev region of HIV-1 is highly diverse, making it difficult to find completely conserved primer binding regions for LAMP primers. To address this, align the primer binding regions of the preliminary primer set selected in **step 3** to the reference HIV-1 sequences downloaded from the Los Alamos database in **step 1**. Identify mutation hotspots located within primer binding regions that would result in primer-template mismatches. Ensure that the primers contain conserved nucleotides at specific positions that are essential for amplification to proceed (3' end of F2/B2 and F3/B3, and 5' end of F1c/B1c. Mutation hotspots are permitted at less essential regions (5' end of F2/B2 and F3/B3, 5' end of the F1c/B1c, and the internal region) where a mismatch will have less effect on amplification. To ensure these criteria, shift the preliminary primers upstream or downstream of the current primer binding site. Recalculate the Tm for each modified primer to ensure that the new primers have similar Tm to the original primers, and that the distance between the modified primer regions must also meet LAMP-specific requirements. The distances between the primer binding regions are calculated from the alignment and the Tm of the modified primers is calculated using Kun's Oligonucleotide Tm calculator (https://arep.med.harvard.edu/kzhang/cgi-bin/myOligoTm.cgi). The calculated Tm is affected by experimental conditions such as the salt concentration and oligo concentration, so it is preferred that Tm be calculated under fixed experimental conditions (oligo concentration at 0.1

µM, sodium ion concentration at 50 mM, magnesium ion concentration at 4 mM).

Similarly, align the selected loop primers to the reference HIV-1 complete genome sequences and modify following the same procedures (shifting the loop primers upstream or downstream of the current primer binding site). Ensure that one of the loop primers fulfills the requirements to be converted into a self-quenching probe and that the internally labeled T and 3' end G/C of the probe region are conserved among the HIV-1 sequences used for the alignment.

The sequences of the HIV-1 genotype B and C LAMP primers and probes designed and used to validate specific detection of Tat/Rev msRNA by RT-LAMP are provided in Table 2.

Table 2: HIV-1 Tat/Rev msRNA specific LAMP primers and probes sequences

Name	**Sequence**
B_F3	GTGTTGCTTTCATTGCCAAG
B_B3	GTCTCTCTCTCCACCTTCT
B_FIP	TGAGGAGCTCTTCGTCGCTGCAAAAGCCTTAGGCATCTC
B_BIP	CAGTCAGACTCATCAAGTTTCTCTCTTCGATTCCTTCGGGCC
B_LF primer	TCTCCGCTTCTTCCTGC
B_LF probe	TCTCCGCTTCTTCC/i6-FAMK/GC
B_LB	AACCCACCTCCCAACCC
C_F3	TCCTTGTAATAAGTGTTATTGTAA
C_B3	CGATTCTTCCGAGCCTGTC
C_FIP	CCGCTTCTTCCTGCCATAGGAATAGCTATCATTGTCTAGTTTGC
C_BIP	CGACGAAGCGCTCCTCCAAGGTTTGGGGTAAGGGTTGCT
C_LF primer	TGCCTAAGCCTTTTGTCTG
C_LF probe	TGCCTAAGCCTTTTGTC/i6-FAMK/G

C_LB	CAGTGAGGATCATCAAAATC

RT-LAMP assay validation using in vitro Tat/Rev RNA transcripts

A gBlock comprising a spliced Tat/Rev sequence (subtype B or C), downstream of a T7 promoter sequence (generated by Integrated DNA Technologies) was used as a template for in vitro transcription using Hi-T7 RNA Polymerase and Ribonucleotide Solution Mix (both from New England Biolabs) in accordance with the manufacturer's instructions. The sequences of the subtype B and subtype C-specific Tat/Rev gBlocks are listed in Table 3.

Table 3: HIV-1 Tat/Rev gBlock sequences

Name	**Sequence**
HIV-1 subtype b gBlock	TAATACGACTCACTATAG**GGGCGGCCGC**ATGGAGCCAGTAGAT CCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAGAC TGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCA AGTTTGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAA GAAGCGGAGACAGCGACGAAGAGCTCCTCAGGACAGTCAGACTC ATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAGCCCCGAGGGG ACCCGACAGGCCCGAAGGAATCGAAGAAGAAGGTGGAGAGAGA GACAGAGACAGATCCGGTCGATTAGTGAATGGATTCTTAGCACTT ATCTGGGACGACCTGCGGAGCCTGTGCCTCTTCAGCTACCACCG CTTGAGAGACTTACTCTTGATTGTAACGAGGATTGTGGAACTTCT GGGACGCAGGGGGTGGGAAGCCCTCAAATATTGGTGGAATCTCC TACAGTATTGGAGTCAGGAACTAAAGAATAG**CCTCGAGG**
HIV-1 subtype c gBlock	TAATACGACTCACTATAG**GGGCGGCCGC**ATGGAGCCAGTAGATC CTAACCTAGAGCCCTGGAACCATCCAGGAAGTCAGCCTAAAACTC CTTGTAATAAGATGTTATTGTAAAAAATGTAGCTATCATTGTCTAG TTTGCTTTCAGACAAAAGGCTTAGGCATTTCCTATGGCAGGAAGA AGCGGAGACAGCGACGAAGCGCTCCTCCAAGCAGTGAGGATCAT CAAAATCTTATATCAAAGCAACCCTTACCCCAAACCCGAGGGGAC CCGACAGGCTCGGAAGAATCGAAGAAGAAGGTGGAGAGCAAGAC AGAGACAGATCCATTCGATTAGTGAACGGATTCTTAGCACTTGCC TGGGACGACCTGCGGAGCCTGTGCCTTTTCAGCTACCACCGATTG AGAGACTTCATATTGGTTGCAGCGAGAGCGGTGGAACTTCTGGG ACGCAGCAGTCTCAGGGGACTACAGAGGGGGTGGGAAGCCCTTA A**CCTCGAGG**

Underlined, T7 promoter sequence; Bold, support sequences; main, HIV-1 Tat/Rev consensus sequence of the mentioned subtype

To eliminate gBlock DNA, in vitro-transcribed Tat/Rev RNA samples were treated with RNase-free DNase I (New England Biolabs) and purified using the Monarch® RNA Cleanup Kit (New England Biolabs). Purified Tat/Rev RNA samples were serially diluted to achieve concentrations of 1000, 500, 250, 125, 50, 20, 10, 5, 1, 0.1 copies of RNA/ 5 µL and used in validation experiments to test the sensitivity of the RT-LAMP reactions. A single RT-LAMP reaction consisted of enzymes (RTx (NEB), Bst 2.0 (NEB), RNasin (Promega), Tat/Rev RNA primer/probe set, and Tat/Rev RNA template to a final reaction volume of 20 µL. Reactions without the RTx enzyme were performed to assess the specificity of the RT-LAMP assay in exclusively amplifying RNA template. The amplification was carried out in a CFX96 Touch Real-Time PCR Detection System thermocycler (BioRad) following a thermal program of continuous 65°C with fluorescence read every 30 sec for 180 cycles, which corresponds to 90 min of amplification time. Complete details of the RT-LAMP reagents and conditions for reaction are listed in Table 4. Positive reactions were identified by the presence of fluorescence curves exceeding the cutoff line.

Table 4: RT-LAMP mastermix composition.

Components and stock conc.	**Volume used**	**Final Conc.**
10X Isothermal Amplification Buffer	2 µL	1X
MgSO4 (100 mM)	1.2 µL	6 mM (8 mM total)
dNTP Mix (10 mM)	2.8 µL	1.4 mM
FIP/BIP Primers (32 µM)	1 µL	1.6 µM
F3/B3 Primers (8 µM)	0.5 µL	0.2 µM
LF primer (doubled as LF self-quenching probe)/LB Primer (16 µM)	0.5 µL	0.4 µM

Bst 2.0 WarmStart DNA Polymerase (NEB Inc., M0538S) (8000 units/mL)	1 µL	8 units
WarmStart RTx Reverse Transcriptase (NEB Inc., M0380S) (15000 units/mL)	0.5 µL	7 units
RNasin® RNase Inhibitor (40 units/ µL)	0.5 µL	20 units
Triton 4%	5 µL	1%
Template	5 µL	
Total Reaction Volume	**20 µL**	

RT-LAMP-based detection of Tat/Rev msRNA in J-Lat 11.1 cells

Latent HIV-1-infected Jurkat cells (clone J-Lat 11.1), which harbor a full integrated HIV-1 subtype B genome with a mutated Env gene and a GFP reporter gene in place of Nef, which requires multiple splicing (Figure 6C), served as a surrogate for inducible reservoir cells. J-Lat 11.1 cells were kindly provided by Eric Verdin of the Gladstone Institute. Single J-Lat 11.1 cells in the background of uninfected donor CD4+T cells were used for the validation of RT-LAMP-based detection of Tat/Rev msRNA. J-Lat 11.1 cells were cultured in complete RPMI-1640 media, supplemented with 7% FBS and 100 µg/ml penicillin-streptomycin, at 37°C in a humidified 5% CO_2 incubator. Primary CD4+ T cells isolated from uninfected donor PBMCs were cultured in RPMI-1640 media supplemented with 10% FBS and 100 µg/ml penicillin-streptomycin at 37°C in a humidified 5% CO_2 incubator. J-Lat 11.1 cells were stimulated with 10 µM of phorbol 12 myristate 13-acetate (PMA) (Sigma) for 12 hrs and primary CD4+T cells were stimulated with 100 ng/mL of PMA (Sigma) and 1 µg/mL of ionomycin (Sigma) for 12 hrs. A Single GFP+ J-Lat 11.1 cell (marking a Tat/Rev msRNA+ cell) was sorted directly into each well of a 96 well white PCR plate (BioRad) containing the RT-LAMP master mix, described in Table 4,

without cells or with an increasing background of activated uninfected donor CD4+ T cells ($5x10^3$, $1x10^4$, $2x10^4$, $4x10^4$, $8x10^4$ cells per reaction). PCR plates were then sealed and RT-LAMP was carried out using a CFX96 Touch Real-Time PCR Detection System thermocycler (BioRad) following a thermal program of continuous 65°C with fluorescence read every 30 s for 180 cycles, which corresponds to 90 min of amplification time. Positive reactions were identified by the presence of fluorescence curves exceeding the cutoff line.

Validating specificity for Tat/Rev ms HIV-1 RNA by RT-LAMP

After 12 hr stimulation of J-Lat 11.1 with PMA (see above) either a single GFP+ or a single GFP- J-Lat 11.1 cell was sorted directly into each well of a 96 well PCR plate containing complete RT-LAMP master mix or the master mix without the reverse transcriptase (RT). PCR plates were then sealed and amplification was carried out with the CFX96 Touch Real-Time PCR Detection System thermocycler (BioRad) following a thermal program of continuous 65°C with fluorescence read every 30 s for 180 cycles, which corresponds to 90 min of amplification time.

Genomic DNA was isolated from PMA-stimulated J-Lat 11.1 cells using the phenol–chloroform-isoamyl alcohol isolation method and ethanol precipitation in the presence of glycogen as a carrier. Isolated DNA was treated with DNase-free Monarch® RNase A (New England Biolabs) to get rid of any RNA contamination. Total RNA was also isolated from PMA-stimulated J-Lat 11.1 cells using Trizol reagent (Sigma) according to the manufacturer's instructions. Isolated RNA was treated with RNase-free DNase I to get rid of any DNA contamination. 100 ng of DNase-treated RNA, 100 ng of RNase-treated DNA and 100 copies HIV-1 plasmid pNL4-3.Luc.R-E- were used as template to perform RT-LAMP on a CFX96 Touch Real-Time PCR Detection System thermocycler (BioRad) following a thermal program of continuous 65°C with fluorescence read every 30 s for 180 cycles, which corresponds to 90 min of amplification time. Positive

reactions were identified by the presence of fluorescence curves exceeding the cutoff line.

SQuHIVLa validation for viral reservoir quantification

To perform SQuHIVLa in custom samples representative of clinical samples with an inducible HIV-1 subtype B reservoir of 0.1, 1 , 10, 20 cells/million CD4+ T cells, J-Lat 11.1 cells and CD4+T cells derived from uninfected donor PBMCs were cultured and stimulated following the same procedure mentioned in the previous section. After stimulation, CD4+ T cells were washed and counted using an automated cell counter (Countess II,Thermo Fisher). A range of 1, 10, 100 or 200 GFP+ J-Lat 11.1 cells were sorted into separate tubes containing activated, uninfected donor CD4+ T cells in RPMI-1640 3% FBS (1 million CD4+ cells/mL). The cells in each tube were then washed, resuspended in Phosphate-buffered saline (PBS) and then serially diluted to 4×10^6 cells/ml, 2×10^6 cells/ml, 1×10^6 cells/ml and 5×10^5 cells/ml in PBS. From each dilution, 5 µL of the cell suspension was distributed to 22–24 wells of a 96-well plate containing 15 µL RT-LAMP master mix (Table 4) corresponding to 20000, 5000, 1250 and 313 cells per well. After the RT-LAMP Reaction, the positive wells at each dilution were scored, and the maximum likelihood method was used to determine the frequency of cells expressing Tat/Rev msRNA using the IUPMStats v1.0 online software[84].

Additional SQuHIVLa validation experiments were performed using peripheral blood samples from people living with HIV (PWH). Primary CD4+ T cells isolated from thawed PBMCS were resuspended in culture RPMI-1640 media supplemented with 10% FBS and 100 µg/ml penicillin-streptomycin and rested for five hrs at 37°C in a humidified, 5% CO_2 incubator. CD4+T cells were then stimulated with 100 ng/mL of PMA and 1 µg/mL of ionomycin (both from Sigma) for 12 hrs. Activated CD4+ T cells were washed in RPMI 1640 media supplemented with 3% FBS, counted at least twice using Countess II (Thermo Fisher) and serially

diluted to 4×10^6 cells/ml, 1×10^6 cells/ml, 2.5×10^5 cells/ml and 6.3×10^4 cells/ml in PBS. From each dilution, 5 µL of the cell suspension was distributed to 22–24 wells of a 96-well plate containing 15 µL RT-LAMP master mix (Table 4). PCR plates were then sealed and RT-LAMP was carried out using a CFX96 Touch Real-Time PCR Detection System thermocycler (BioRad) following a thermal program; incubation at 45°C for 60 mins (determined based on the results depicted in Fig. 3E) followed by continuous 65°C with fluorescence read every 30 s for 180 cycles, which corresponds to 90 min of amplification time. After the RT-LAMP reaction, the positive wells at each dilution were scored, and used to determine the frequency of cells expressing Tat/Rev msRNA, using the IUPMStats v1.0 online software[84], which uses maximum likelihood statistics.

When a limited number of PBMCs were available, activated CD4+ T cells were serially diluted to 2×10^6 cells/ml, 1×10^6 cells/ml, 2.5×10^5 cells/ml and 6.3×10^4 cells/ml in PBS and, 5 µL of the cell suspension was distributed to 22–24 wells of a 96-well plate corresponding to 10000, 5000, 1250 and 313 cells per well.

Statistical analysis

All graphs were generated and statistical analyses were performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). In order to calculate the lowest amount of Tat/Rev ms RNA in a sample that can be consistently detected with 95% probability (LOD 95%), probit analysis was used. The Probit was calculated by the Excel function [5+NORMSINV(P)], where P was the hit-rate (proportion of positive reactions) and 6.64 probit value (1.64 for 95% limit, +5 for probit scale) was used to extrapolate LOD 95% RNA copies. The effect of background cells on RT-LAMP amplification time was determined using Ordinary One-way ANOVA followed by Dunnett's multiple comparisons test. Coefficient of variation (CV) was calculated to determine the reproducibility of SQuHIVLa assay. Normality of the calculated

inducible HIV-1 reservoir size (Fig. 4D) using SQuHIVLa was tested with the Shapiro-Wilk test. All the data passed the normality test which is why correlations were performed using the Pearson test. Accuracy percentage (AP) in Fig 4D was calculated using ABS function in Excel. Inducible HIV-1 subtype C reservoir size quantified from infected individuals with different biological sex were compared utilizing two-tailed unpaired t-test.

Example 1: A LAMP primer/probe set designed for exclusive detection of HIV-1 Tat/Rev msRNA

The successful design of a LAMP primer/probe set for specific LAMP detection of HIV-1 Tat/Rev msRNA, depended on a set of guidelines as depicted in Fig 1. The most critical criterion to ensure precise detection of Tat/Rev msRNA, excluding intron-containing Tat/Rev HIV-1 genomic DNA, was that the primer binding sites are exon-spanning. Using HIV-1 subtype B as reference, we designed primer/probe sets as depicted in the flowchart (Fig. 1). We retrieved and aligned ten complete genome sequences of HIV-1 subtype B from different years (1983-2005) and various geographical locations to generate an *in silico*-spliced Tat/Rev HIV-1 DNA consensus sequence. Using the PrimerExplorer V5 software and the consensus Tat/Rev HIV-1 DNA FASTA file, we generated a list of preliminary sets of LAMP primers from which we selected primer sets where either the F2 binding region was within nucleotide position 125 to 240 or the B2 binding region was within nucleotide position 190 to 305, to ensure that the splice site (215^{th} nucleotide) was located within the forward loop (5'F2 to 3'F1) or backward loop (5'B2 to 3'B1). This criterion ensures that unstable loop formation resulting from primer binding to intron-containing Tat/Rev DNA, would inhibit isothermal amplification, thereby resulting in the exclusive detection of Tat/Rev msRNA (Fig. 2A). Additionally, we generated two loop primers using the PrimerExplorer V5 software and aligned these and the other six LAMP primers to the ten HIV-1 genome sequences to assess primer-target sequence complementarity (Fig. 2B). Given HIV-1's high

genetic diversity, it is impossible to ensure that the primer binding regions in patient-specific viral RNA are devoid of mutation hotspots. However, we strived to ensure that the mutation hotspots are located at positions such as the 5' ends of F3, B3, F2, B2, and 3' ends of F1c, B1c primer binding regions (Fig. 2B). A set of primers with mismatches at these positions, whilst still meeting all the LAMP primer criteria such as melting temperature and distance between primer binding sites, would have the least impact on amplification (Methods). Furthermore, we converted the loop forward primer (LF) into a self-quenching probe to enable sequence-specific, real-time detection and quantification of LAMP amplicons (Methods).

Example 2: Highly sensitive and specific detection of Tat/Rev msRNA by RT-LAMP

To assess the sensitivity of RT-LAMP in detecting Tat/Rev msRNA, we used serial dilutions of msRNA, *in vitro* transcribed from an intron-free Tat/Rev DNA template, driven by a T7 promoter (Fig. 3A). As few as 50 copies of RNA were detected within 30.46 minutes (±7.21 minutes) using RT-LAMP in >97% of all reactions, with a Limit of Detection-95% at 31 copies (Fig. 3B, D, E). At lower RNA copy numbers, RT-LAMP was less efficient in amplifying Tat/Rev msRNA shown by longer time to result and increased variability between reactions (Fig. 3C). In experiments to assess the specificity of Bst 2.0 polymerase enzyme for Tat/Rev msRNA, we performed LAMP reactions with the required Bst 2.0 polymerase enzyme in the absence of the reverse transcriptase enzyme, RTx (Methods). Minimal and inconsistent amplification was observed at high copies of RNA ($\geq$ 250 copies), suggesting some basal reverse transcriptase activity, which manufacturer validation experiments have also shown. However, the RT enzyme is necessary for robust and consistent amplification of RNA templates (Figure 6A and B).

Subsequently, to determine the sensitivity of RT-LAMP in detecting Tat/Rev msRNA directly from cells, we used J-Lat 11.1 cells, a

Jurkat cell line harboring a latent but inducible full-length HIV-1 genome that expresses green fluorescence protein (GFP), as a product of msRNA transcripts (Fig. 6C), upon activation. We sorted single GFP+ cells (Fig. 6D) into RT-LAMP reaction plates in the presence or absence of PMA-stimulated uninfected donor CD4+ T cells (Fig. 3F). In the absence of CD4+ T cell background, a single GFP+ cell was detected in 95.83% of all RT-LAMP reactions (Fig. 3G), decreasing to 83.33% positivity in a background of $2x10^4$ cells, however the presence of cell background did not significantly impact the amplification time of RT-LAMP (mean amplification time ranging from 31.26 mins for no cell background to 42.63 mins in a background of $8x10^4$ cells) (Fig. 3H and I).

Importantly, in another set of validation experiments we observed specific amplification of Tat/Rev msRNA and not intron-containing Tat/Rev genomic DNA. Minimal to no amplification was observed when RT-LAMP reactions were performed without the reverse transcriptase enzyme in a range of validation samples including; GFP- J-Lat 11.1 cells that do not express msRNA (Fig. 4A, Fig. 7); DNase-treated J-Lat 11.1 RNA and RNase-treated J-Lat 11.1 DNA or using HIV-1 plasmid as template (Fig. 4B). Overall, we demonstrate the rapid, sensitive and specific detection of cells expressing Tat/Rev msRNA by RT-LAMP and sought to use this assay for quantifying the inducible HIV-1 reservoir, hereafter referred to as SQuHIVLa (Specific Quantification of Inducible HIV-1 reservoir by LAMP).

Example 3: Accurate and reproducible quantification of the inducible viral reservoir using SQuHIVLa

In the next set of experiments, we sought to assess the reproducibility of SQuHIVLa in quantifying the inducible viral reservoir. To model HIV-1 reservoir samples, we generated a panel of four test samples comprising activated GFP+ J-Lat 11.1 cells (0.1, 1, 10 and 20 cells) sorted into a suspension of one million CD4+ T cells. Cells from each test sample were then distributed into RT-LAMP reaction plates in limiting dilution

format, which allows quantification by maximum likelihood statistics (Fig.4C, Methods). The observed and predicted viral reservoir size measurement were highly precise at frequencies greater than 20 GFP+ cells/million CD4+ T cells with a coefficient of variation (CV) of 6.61%). The CV increased to 60.61% at 0.1 cell GFP+ cells/million CD4+T cells (Fig. 4D). Furthermore, the observed and predicted viral reservoir measurements correlated significantly with a good accuracy with mean accuracy percentage (AP) of 85% for reservoir size greater than 1 GFP+ cell/million CD4+ T cells (Fig. 4E).

To further validate SQuHIVLa, we applied the assay to quantify the inducible viral reservoir in primary CD4+ T cells obtained from people with HIV-1 subtype B (PLWHB) on suppressive antiretroviral therapy. Following 12-hour PMA/ionomycin stimulation, which enhances transcriptional activation of inducible proviruses, whole CD4+ T cells were distributed in a limiting dilution format and Tat/Rev msRNA transcripts were amplified using RT-LAMP (Fig. 4F). The frequency of cells expressing Tat/Rev msRNA was then determined by maximum likelihood estimation. We observed sub-optimal Tat/Rev msRNA amplification in primary CD4+ T cells, which was resolved through optimization of the sample incubation time at 45°C prior to isothermal amplification at 65°C. As the viral reservoir size in three independent donors did not increase with incubation times exceeding one hour, we incorporated an incubation time of one hour at 45°C into the SQuHIVLa protocol when quantifying reservoir in primary CD4+ T cells (Fig. 4G). The mean inter-assay CV, 9.58% (Range:7.2%-12.23%) was determined from three independent SQuHIVLa experiments using CD4+ T cells from three PLWHB demonstrating high reproducibility of SQuHIVLa in quantifying the inducible HIV-1 reservoir as depicted in Fig. 4H.

Example 4: Adaptation of SQuHIVLa for non-B HIV-1 Subtypes

To determine the feasibility of using one primer-probe set for different HIV-1 subtypes, we aligned the subtype B Tat/Rev msRNA LAMP

primer-probe sequences to genome sequences of HIV-1 subtypes C and A. A heatmap of the mismatch score, corresponding to the relative quantity of matching nucleotides for each primer binding region, revealed poor compatibility of the subtype B LAMP primer-probe sets for detection of Subtypes C and A Tat/Rev msRNA (Fig. 8). Several mismatches were present at sites critical for the LAMP reaction. Therefore, to specifically detect Tat/Rev msRNA from people with HIV-1 subtype C, globally the most prevalent subtype, we designed a new set of LAMP primers and probe (Fig. 5A) following the aforementioned guidelines (Figure 1 and methods) and fulfilling the requirements to ensure specific amplification of subtype C Tat/Rev msRNA. The new primer-probe set demonstrated similar sensitivity in detecting *in-vitro* HIV-1 C Tat/Rev msRNA transcripts compared to the HIV-1 subtype B primer/probe set. In all experiments performed, 50 copies of Tat/Rev HIV-1 subtype C msRNA were detected in >97% reactions (Fig. 5B) with a mean amplification time of 38.19 minutes (±7.47 minutes) (Fig. 5C) and a LOD-95 % of 45 copies (Fig. 5D, E), similar to that observed for Subtype B Tat/Rev msRNA.

We then assessed the performance SQuHIVLa across a panel of clinical samples from 19 individuals with HIV-1 Subtype C on fully suppressive antiretroviral therapy (17 chronically infected and 2 elite controllers). Tat/Rev msRNA was detectable in all samples and the frequency of cells expressing Tat/Rev msRNA ranged from 10.12-98.08 cells per million CD4+ T cells (Fig. 5F). Interestingly, in the chronically-infected individuals, the inducible viral reservoir was notably higher in females (n=10) than in males (n=7) (P=0.0202) (Fig. 5G). Additionally, viral reservoir quantification was also possible for the two HIV-1 Subtype C elite controllers. Univariate correlation analyses did not reveal significant association between the frequency of cells expressing Tat/Rev msRNA and CD4+ T cell counts or pre-ART plasma HIV-1 RNA (Fig. 9) Overall, these findings demonstrate that SQuHIVLa is highly adaptable and has great

potential for application in studies involving people with HIV-1, with various clinical characteristics.

Discussion

A rare fraction of intact, replication-competent provirus persists in PLWH despite potent suppressive antiretroviral therapy, constituting a latent reservoir of cells capable of initiating viral replication and viral rebound upon cART interruption. Several state-of-the-art technologies have been used to assess HIV-1 persistence at the level of viral DNA, RNA, protein or replication, both quantitatively and qualitatively. The majority of persistent provirus at the DNA level is replication incompetent or defective, and has been shown to be remarkably stable over years of suppressive antiretroviral therapy. However, recent work has demonstrated that defective HIV-1 proviruses are still capable of expressing unspliced viral RNA and defective viral proteins. While this is considered a measure of transcription and, to some extent translation-competence, this measure of viral persistence does not contribute to the viral reservoir of intact, inducible and replication-competent proviruses. Emerging work has demonstrated that Tat/Rev msRNA expression, which correlates with plasma HIV-1 RNA upon latency reversal, may be a relevant and promising indicator of the inducible, replication-competent viral reservoir. Existing approaches to quantify the levels of Tat/Rev msRNA utilize real time RT-qPCR or RT-ddPCR on bulk Total cellular RNA. These methods are highly sensitive and specific for the targeted viral RNA transcript. Certain RNA induction assays directly probe for Tat/Rev msRNA using whole cells, without RNA extraction, which may substantially simplify the workflow in that regard. However, semi-nested RT-qPCR is necessary to amplify the low copies of Tat/Rev msRNA transcripts to detectible and quantifiable levels. Furthermore, it should be noted that the direct whole cell input and semi-nested RT-qPCR method of quantification poses several limitations, with direct consequences on the accuracy of the assay endpoint. An important

limitation, is the increased likelihood of detecting Tat/Rev within genomic HIV-DNA, which would introduce assay false positives, leading to overestimated measurements of the inducible viral reservoir. Moreover, semi-nested RT-qPCR approaches, performed in limiting dilution, are low throughput and considerably increase cross-contamination risks, the latter leading to false measurements. To address these two critical caveats of Tat/Rev msRNA RT-qPCR method of detection, we leveraged the superior qualities of RT-LAMP to develop SQuHIVLA, a rapid, sensitive, highly accurate and specific assay for Tat/Rev msRNA detection and quantification. SQuHIVLA detects and quantifies HIV-1-infected cells that spontaneously or inducibly express Tat/Rev msRNA and provides a first demonstration of LAMP technology for HIV-1 viral reservoir assessment and quantification.

In developing LAMP primers for the exclusive detection of Tat/Rev msRNA, it was essential to design primers that were exon-spanning. The Tat/Rev intron, approximately 2 kb, if incorporated into the forward or backward loop, during amplification, would decrease the stability of the formed loops, thereby inhibiting further target amplification and the fluorescent self-quenching loop primer, which is used as a reporter for specific target amplification, would be non-functional. We have developed a comprehensive set of guidelines to follow when designing Tat/Rev exon-spanning LAMP primers, which must fulfil crucial target-specific criteria in addition to the standard LAMP primer requirements. Designing LAMP PCR primers and a probe to bind within the Tat/Rev genomic region is extremely challenging due to high HIV-1 genetic diversity and the need to identify eight relatively conserved primer binding regions for the LAMP reaction. A universal LAMP primer/probe set for the broad detection of Tat/Rev msRNA expressed from multiple HIV-1 subtypes is quite likely not feasible. It may be possible to multiplex LAMP primer/probe sets for different HIV-1 subtypes into one LAMP reaction. However, this will be challenging to achieve without complex molecular design and will require an extensive number of optimizations.

The highly sensitive detection of Tat/Rev msRNA within half an hour of target amplification is revolutionary and superior to existing semi-nested RT-qPCR methods. We capitalized on this rapid amplification, high sensitivity and specificity of RT-LAMP, performed in a single reaction, to enhance the method of detecting and quantifying cells expressing Tat/Rev msRNA upon activation (SQuHIVLa). We have demonstrated, as a proof of concept, RT-LAMP-based inducible viral reservoir assessment in CD4+ T cells from a diverse group of individuals with HIV-1 subtype B and C on suppressive antiretroviral therapy. Although limited in number, our data indicate good sensitivity and further assessment of the assay using cells from individuals with low or ultra-low viral reservoir size is warranted. In our cohort of individuals with HIV-1 subtype C, treated during chronic infection stage, the mean frequency of cells expressing Tat/Rev msRNA after *in vitro* treatment with PMA/Ionomycin was significantly higher in women compared to men. Few studies have investigated sex differences in HIV-1 reservoir size, mostly determined by methods to quantify HIV-1 DNA and integrated HIV DNA, which have been reported to be comparable in men and women. Studies that have assessed the viral transcription and replication by quantitating levels of cell-associated unspliced HIV-1 RNA and viral outgrowth have revealed a smaller inducible viral reservoir in women, which contradicts our findings. This discrepancy however could be explained by differences in the endpoints, unspliced versus multiply spliced viral RNA transcripts measured by the different assays, which reflect different capacities of viral inducibility. Furthermore, reservoir quantification of viral outgrowth is often an underestimation due to suboptimal induction of majority of proviruses. It is likely that the frequency of inducible, replication competent proviruses is truly small in women, or poorly inducible. Indeed, HIV-1 inducibility in women, is potentially influenced by estrogen, which has been demonstrated to repress HIV-1 transcription in latency models, in vitro infection systems, and primary cells, implicating a direct role for sex hormones mediating sex

differences in reservoir maintenance and dynamics. Further research with a larger sample size and mechanistic studies are needed to better understand any potential sex-specific differences in the inducible reservoir size and the implications thereof.

Amid the rise of explorative strategies to eliminate the inducible viral reservoir in individuals with HIV-1, reliable, sensitive and scalable assays are essential to determine the efficacy of putative intervention strategies. Moreover, it will be important for future studies to assess large cohorts in Sub-Saharan Africa, which bears a disproportionate burden of HIV-1. This also points to the need for the applicability of any future viral reservoir quantification assays in resource-constrained settings.

RT-LAMP is highly sensitive, capable of detecting low copy numbers of RNA targets, and can provide quantitative results through real-time monitoring. Furthermore, it is potentially less expensive, user-friendly, and compatible with a variety of sample types. These features make RT-LAMP a promising tool for quantitative analysis in various fields, including HIV-1 molecular diagnostics and viral load monitoring. Here, we have demonstrated that the exclusive detection of Tat/Rev msRNA, a proximal surrogate marker for inducible, replication-competent HIV-1, is possible using SQuHIVLA. Further research and collaboration with biomedical industries is needed to develop certified high-throughput assays for HIV-1 and other pathogens based on the principles of SQuHIVLa.

Example 5. Single cell SQuHIVLa

This example describes an exemplary embodiment of a method of the invention wherein the HIV-1 msRNA is amplified within individual cells *in situ,* and wherein positive cells are quantified by flow cytometry. Single cell quantitation allows accurate quantitation of smaller reservoirs, especially post-intervention or in individuals with naturally small reservoirs, and suffers less from background signals.

In this example the RT-LAMP reaction is performed on a sample comprising cells in which Tat/Rev multiply spliced HIV-1 RNA is to be detected using the primer set specific for Tat/Rev multiply spliced HIV-1 RNA as described above and determining whether the cells in the sample comprises an amplification product of the amplification reaction as described above. RT-LAMP-based detection of Tat/Rev msRNA in this example is performed in J-Lat 11.1 cells.

J-Lat 11.1 cells were stimulated with PMA for 12 hours as described above. The cells were washed twice with PBS. Following the washing, the cells were stained with Fixable Viability Dye eFluor™ 780 (Invitrogen) for 20 minutes on ice. After staining, the cells were washed twice with PBS and then treated with ice-cold 80% methanol for 10-15 minutes on ice. The cells were then washed two times with PBS and finally resuspended in PBS. The RT-LAMP reaction was performed with the RT-LAMP mastermix composition as described in Table 4, except that the self-quenching probe was replaced with a molecular beacon. The final concentration of the molecular beacon was 0.4 μM.

The sequence of the molecular beacon was 5'-CCGGCGCTCTCCGCTTCTTCCTGCGCGCCGG-3'. The underlined part (TCTCCGCTTCTTCCTGC) represents the sequence of the loop forward primer and forms the target-specific sequence of the molecular beacon. The sequence of the loop forward primer is flanked by random nucleotides containing C/G bases to form the complete sequence of the molecular beacon. The 5' and 3' flanking nucleotide sequences are self-complementary, allowing the formation of a stem-loop structure when unbound in the absence of the target sequence. When the target sequence is present, and the target-specific sequence binds to its target, the 5' end (with the fluorophore) and the 3' end (with the quencher) separate whereby the molecular beacon generates a fluorescence signal. The fluorophore 6-FAM (IDT) was attached to the 5' end. The quencher 3IABkFQ (IDT) was attached to the 3' end.

The cells as resuspended in PBS were used as the input sample for the isothermal amplification reaction. The isothermal amplification was carried out at 65°C for 30 minutes. After RT-LAMP, the complete mastermix and cells were transferred from the PCR well to a FACS tube containing 500 µL of PBS. The cells were then analyzed by flow cytometry for RT-LAMP amplification based on the molecular beacon signal. Flow cytometry was performed on a BD LSRFortessa™ Cell Analyzer (BD Bioscience), using standard instrument settings.

The results are displayed in Figure 16, wherein it is shown that the control cells that were not subjected to the RT-LAMP reaction (minus SQuHIVLa) are positioned in Q4, whereas cells that were subjected to the RT-LAMP reaction (plus SQuHIVLa) are positioned in Q3. This shift is due to successful amplification of Tat/Rev multiply spliced HIV-1 RNA within individual J-Lat 11.1 cells.

Claims

1. A method for determining whether a sample comprises inducible HIV-1, the method comprising performing a reverse transcription, loop-mediated isothermal amplification (RT-LAMP) reaction with said sample with a primer set specific for Tat/Rev multiply spliced (ms) HIV-1 RNA and determining whether the sample comprises an amplification product of the RT-LAMP reaction, wherein the binding sites for the F2 and F1 primer or for the B2c and B1c primer of said primer set span a region in said Tat/Rev ms HIV-1 RNA that overlaps with the splice site of the intron interrupting the Tat and Rev coding sequences.

2. A method of detecting an amplification product in a sample, the method comprising performing an RT-LAMP reaction with said sample with a primer set specific for Tat/Rev multiply spliced HIV-1 RNA and determining whether the sample comprises an amplification product of the amplification reaction, wherein the binding sites for the F2 and F1 primer or for the B2c and B1c primer of said primer set span a region in said Tat/Rev ms HIV-1 RNA that overlaps with the splice site of the intron interrupting the Tat and Rev coding sequences.

3. The method of claim 1 or 2, wherein the primer binding sites for the F2 and B2c primers are located in the region defined by nucleotide positions 55 to 375 of the sequence of said Tat/Rev ms HIV-1 RNA,

preferably wherein the primer binding site for the F2 primer is located in the region defined by nucleotide positions 125 to 240 or preferably wherein the primer binding site for the B2c primer is located in the region defined by nucleotide position 190 to 305 of the sequence of said Tat/Rev ms HIV-1 RNA,

preferably wherein the distance between the F1 and F2 regions and/or between the B1c and B2c regions is at least 22 nucleotides.

4. Method according to any of the preceding claims, wherein said primer set comprises:

i) a forward inner primer (FIP) comprising at least two regions F2 and F1c, wherein F1c is linked to the 5'-side of F2, and wherein F2 is a region having a nucleotide sequence complementary to a region F2c in said Tat/Rev ms HIV-1 RNA sequence, and F1c is a region having substantially the same nucleotide sequence as a region F1c located at the 5'-side of the region F2c in said Tat/Rev ms HIV-1 RNA sequence;

ii) a backward inner primer (BIP) comprising at least two regions B2 and B1c, wherein B1c is linked to the 5'-side of B2, and wherein B2 is a region having a nucleotide sequence complementary to a region B2c in a complementary chain synthesized from said Tat/Rev ms HIV-1 RNA sequence with the forward inner primer as the origin of synthesis, and B1c is a region having substantially the same nucleotide sequence as a region B1c located at the 5'-side of the region B2c in said complementary chain;

iii) a forward outer primer (F3) having a nucleotide sequence complementary to a region F3c located at the 3'-side of the region F2c in said Tat/Rev ms HIV-1 RNA sequence;

iv) a backward outer primer (B3) having a nucleotide sequence complementary to a region B3c located at the 3'-side of the region B2c in said complementary chain;

v) a forward loop primer (LF) having a nucleotide sequence complementary to a region LFc in said Tat/Rev ms HIV-1 RNA sequence located between the regions F1c and F2c;

vi) a backward loop primer (LB) having a nucleotide sequence complementary to a region LBc located between the regions B1c and B2c in said complementary chain.

5. Method according to claim 4, wherein said sequence of said Tat/Rev ms HIV-1 RNA is the consensus sequence of *in silico* spliced

Tat/Rev ms HIV-1 RNA sequences of a plurality of distinct HIV-1 isolates of a single HIV-1 subtype.

6. Method according to claim 4, wherein the LF or LB loop primer comprises a cytosine (C) or guanine (G) residues at its 3′-end that represent a conserved nucleotide in said consensus nucleic acid sequence, and wherein the LF and/or LB loop primers comprise a thymine (T) residue at the second or third position from the 3′-end, preferably wherein the LF or LB loop primer is a self-quenching probe or molecular beacon.

7. Method according to any one of the preceding claims, wherein the reverse transcription and loop-mediated isothermal amplification reactions are performed in a single reaction mixture comprising template RNA, dNTP's, a thermostable reverse transcriptase, and a DNA polymerase having strand displacement activity, more preferably (Bst) DNA polymerase.

8. Method according to any one of claims 4-7, wherein said consensus sequence is a consensus sequence as set forth in any one of Figures 10-15 and sequences having at least 80% sequence identity thereto.

9. A method for the detection of inducible HIV-1 in a subject, preferably a subject receiving or having received antiretroviral therapy (ART), the method comprising the steps of:
a) providing a sample of $CD4^+$ T cells from said subject;
b) stimulating said $CD4^+$ T cells in said sample to thereby provide a sample of activated $CD4^+$ T cells;
c) performing the method of any one of claims 1-8 on said sample of activated $CD4^+$ T cells, and detecting an amplification product of the RT-LAMP reaction as an indication of the presence of inducible HIV-1 in said sample or in said subject.

10. A method of quantifying the inducible HIV-1 reservoir in a subject receiving or having received antiretroviral therapy (ART), the method comprising performing the method of claim 9, wherein said step b) further comprises preparing serial volumetric dilutions of said sample of activated $CD4^+$ T cells, and wherein said step c) is followed by the step of quantifying the amount of Tat/Rev multiply spliced HIV-1 RNA-containing $CD4^+$ T cells in said sample as a ratio of the total number of $CD4^+$ T cells in the sample provided in said step a) to thereby provide an indication of the size of the inducible HIV-1 reservoir in said subject.

11. A method of quantifying the inducible HIV-1 reservoir in a subject receiving or having received antiretroviral therapy (ART), the method comprising performing the method of claim 9 on individual cells from said sample of activated $CD4^+$ T cells provided in said step b), and wherein said step c) is followed by the step of quantifying the amount of Tat/Rev multiply spliced HIV-1 RNA-containing $CD4^+$ T cells in said sample as a ratio of the total number of $CD4^+$ T cells in the sample provided in said step a) to thereby provide an indication of the size of the inducible HIV-1 reservoir in said subject.

12. Method according to claim 11, wherein said primer set comprises an LF or LB loop primer, and wherein said LF or LB loop primer is labeled with a fluorescent dye in the 5′ end and a quencher molecule in the 3′ end.

13. Method according to any one of claims 9-12, wherein stimulating said $CD4^+$ T cells in said sample is performed by exposing said cells to an effective amount of a compound selected from anti-CD3 antibody, anti-CD28 antibody, anti-histone deacetylase (HDAC) antibody, apicidin, 5-azacytidine (5-Aza), 5-Aza-CdR, azelaic bishydroxamic acid (ABHA), bryostatin, butyric acid, m-carboxycinnamic acid bis-hydroxamide (CBHA), dacinostat (LAQ824), 12-deoxyphorbol 13-phenylacetate (DPP) depudecin, entinostat

(MS-275), 5-fluoro-2'-deoxycytidine, glutamate, hexamethylbisacetamide (HMBA), hydralazine, IL-7, IL-2, IL-1ß, ingenol mebutate, 3-caproyl-ingenol, ingenol 3,20-dibenzoate, ionomycin, M344 (D237), midostaurin (PKC412), oxamflatin, panobinostat (LBH589), phenylbutyrate, phenylacetate, phorbol myristate acetate (PMA), N-phthalyl-L-tryptophan (RG108), phytohemagglutinin (PHA), procaine, procainamide, prostratin, pyroxamide, romidepsin (FK228), scriptaid, sodium butyrate, suberic bishydroxamic acid (SBHA), suberoylanilide hydroxamic acid (SAHA), tacedinaline (CI-994), trapoxin B, trichostatin A, tumor necrosis factor-α (TNFα), valproic acid, zebularine, and combinations thereof.

14. Method according to any one of the preceding claims wherein the method of any one of claims 1-8 is performed on whole cells, preferably on activated $CD4^+$ T cells.

15. Use of a set of primers comprising:

- a forward outer primer having the sequence GTGTTGCTTTCATTGCCAAG;
- a backward outer primer having the sequence GTCTCTCTCTCCACCTTCT;
- a forward inner primer having the sequence TGAGGAGCTCTTCGTCGCTGCAAAAGCCTTAGGCATCTC;
- a backward inner primer having the sequence CAGTCAGACTCATCAAGTTTCTCTCTTCGATTCCTTCGGGCC;
- a forward loop primer having the sequence TCTCCGCTTCTTCCTGC; and
- a backward loop primer having the sequence AACCCACCTCCCAACCC,

for amplifying Tat/Rev ms HIV-1 DNA from HIV-1 subtype B.

16. Use of a set of primers comprising:

- a forward outer primer having the sequence TCCTTGTAATAAGTGTTATTGTAA;
- a backward outer primer having the sequence CGATTCTTCCGAGCCTGTC;
- a forward inner primer having the sequence CCGCTTCTTCCTGCCATAGGAATAGCTATCATTGTCTAGTTTGC;
- a backward inner primer having the sequence CGACGAAGCGCTCCTCCAAGGTTTGGGGTAAGGGTTGCT;
- a forward loop primer having the sequence TGCCTAAGCCTTTTGTCTG, and
- a backward loop primer having the sequence CAGTGAGGATCATCAAAATC,

for amplifying Tat/Rev ms HIV-1 DNA from HIV-1 subtype C.

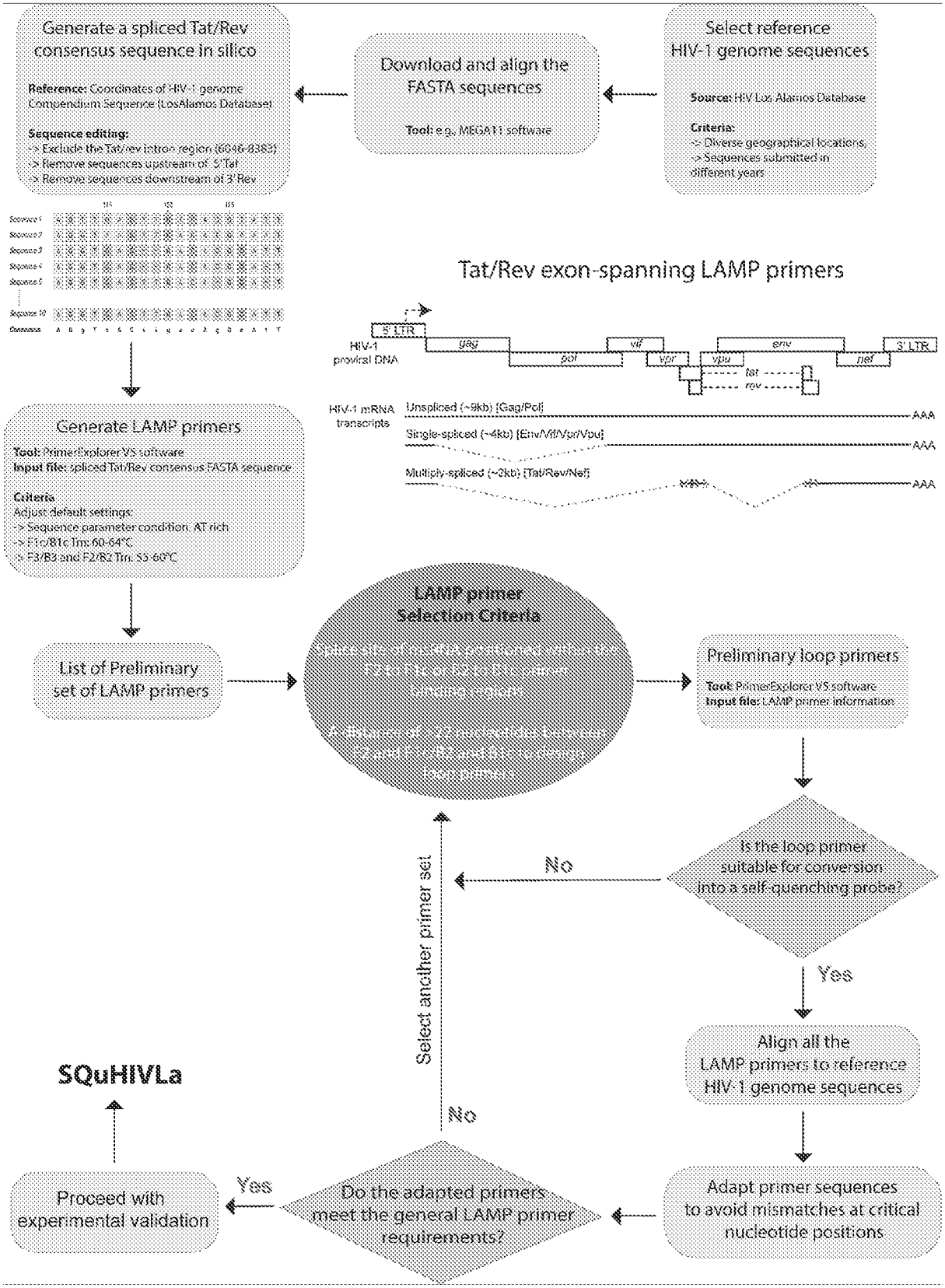
Generate a spliced Tat/Rev consensus sequence in silico
Reference: Coordinates of HIV-1 genome Compendium Sequence (LosAlamos Database)
Sequence editing:
-> Exclude the Tat/rev intron region (6046-8382)
-> Remove sequences upstream of 5' Tat
-> Remove sequences downstream of 3' Rev
Download and align the FASTA sequences
Tool: e.g., MEGA11 software
Select reference HIV-1 genome sequences
Source: HIV Los Alamos Database
Criteria:
-> Diverse geographical locations,
-> Sequences submitted in different years
Tat/Rev exon-spanning LAMP primers
5' LTR
gag
pol
vif
vpr
vpu
env
tat
rev
3' LTR
HIV-1 proviral DNA
HIV-1 mRNA transcripts
Unspliced (~9kb) [Gag/Pol]
Single-spliced (~4kb) [Env/Vif/Vpr/Vpu]
Multiply-spliced (~2kb) [Tat/Rev/Nef]
AAA
Generate LAMP primers
Tool: PrimerExplorer V5 software
Input file: spliced Tat/Rev consensus FASTA sequence
Criteria
Adjust default settings:
-> Sequence parameter condition: AT rich
-> F1c/B1c Tm: 60-64°C
-> F3/B3 and F2/B2 Tm: 55-60°C
List of Preliminary set of LAMP primers
LAMP primer Selection Criteria
Preliminary loop primers
Tool: PrimerExplorer V5 software
Input file: LAMP primer information
Is the loop primer suitable for conversion into a self-quenching probe?
No
Yes
Select another primer set
Align all the LAMP primers to reference HIV-1 genome sequences
Adapt primer sequences to avoid mismatches at critical nucleotide positions
Do the adapted primers meet the general LAMP primer requirements?
No
Yes
Proceed with experimental validation
SQuHIVLa

Fig. 1

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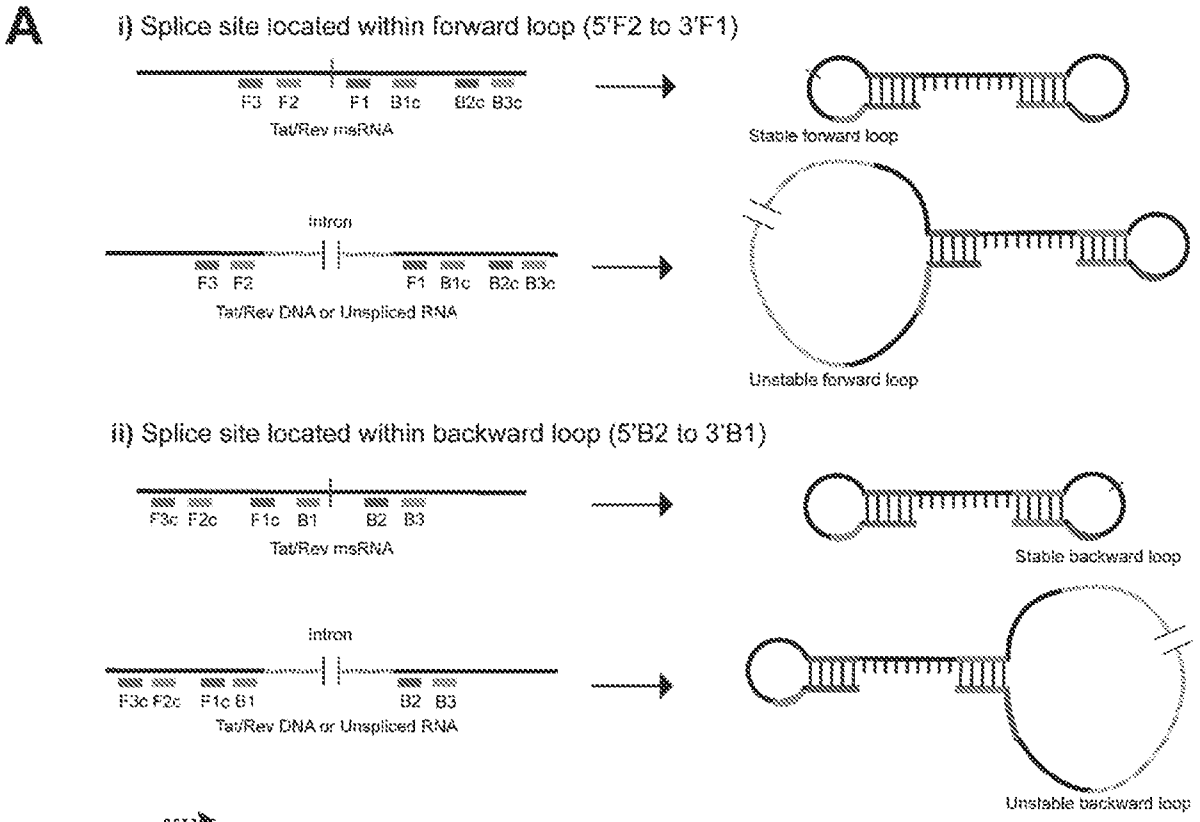
Fig. 2



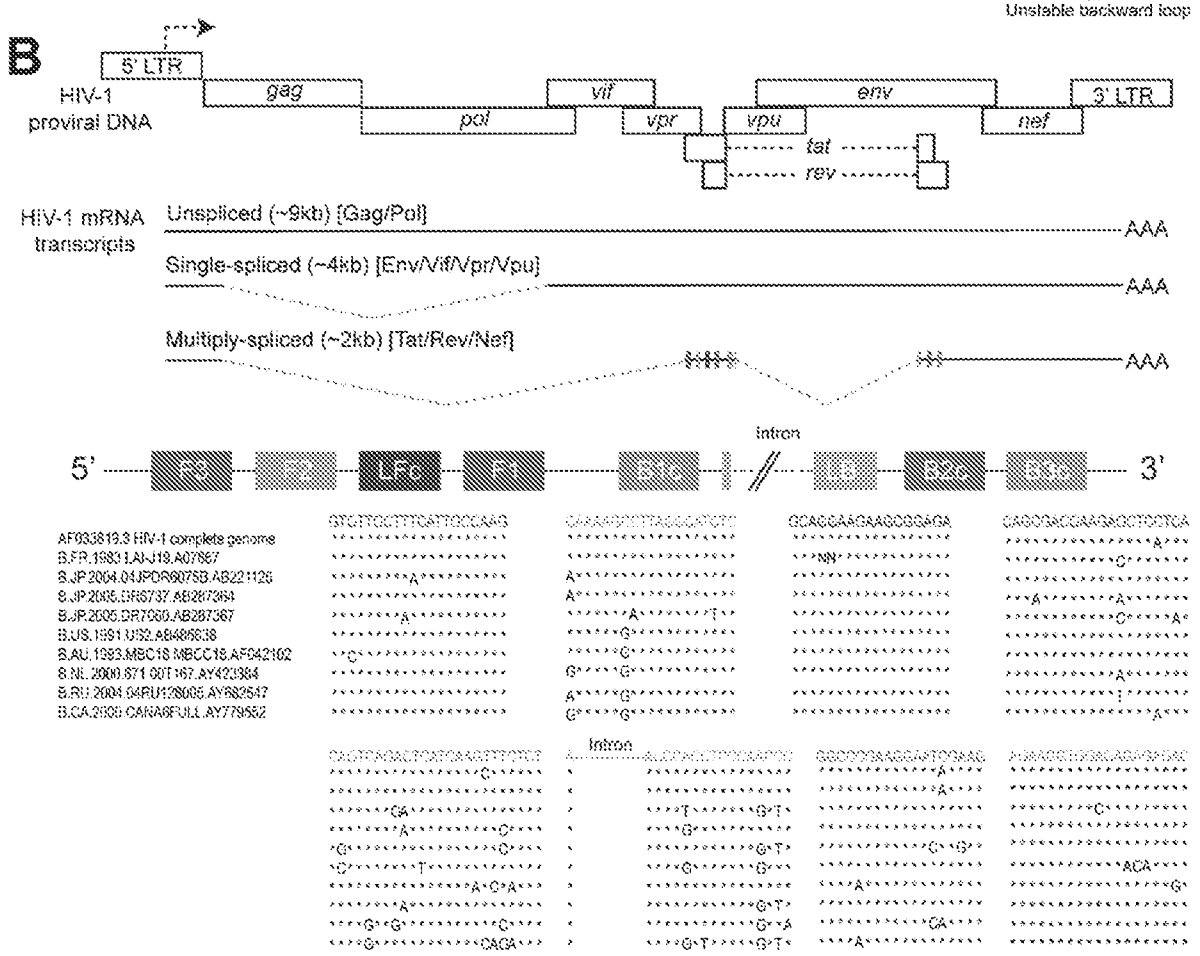


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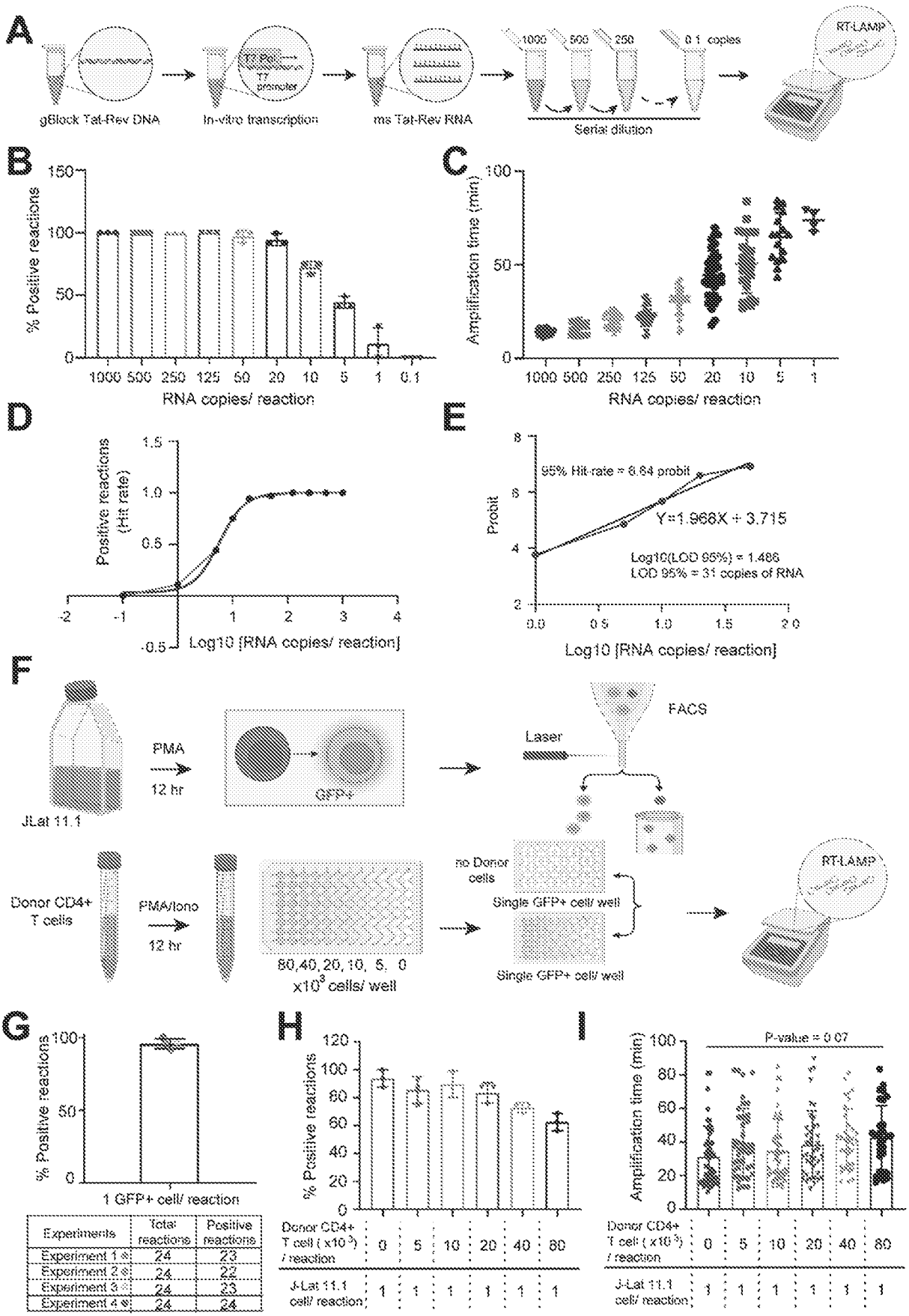
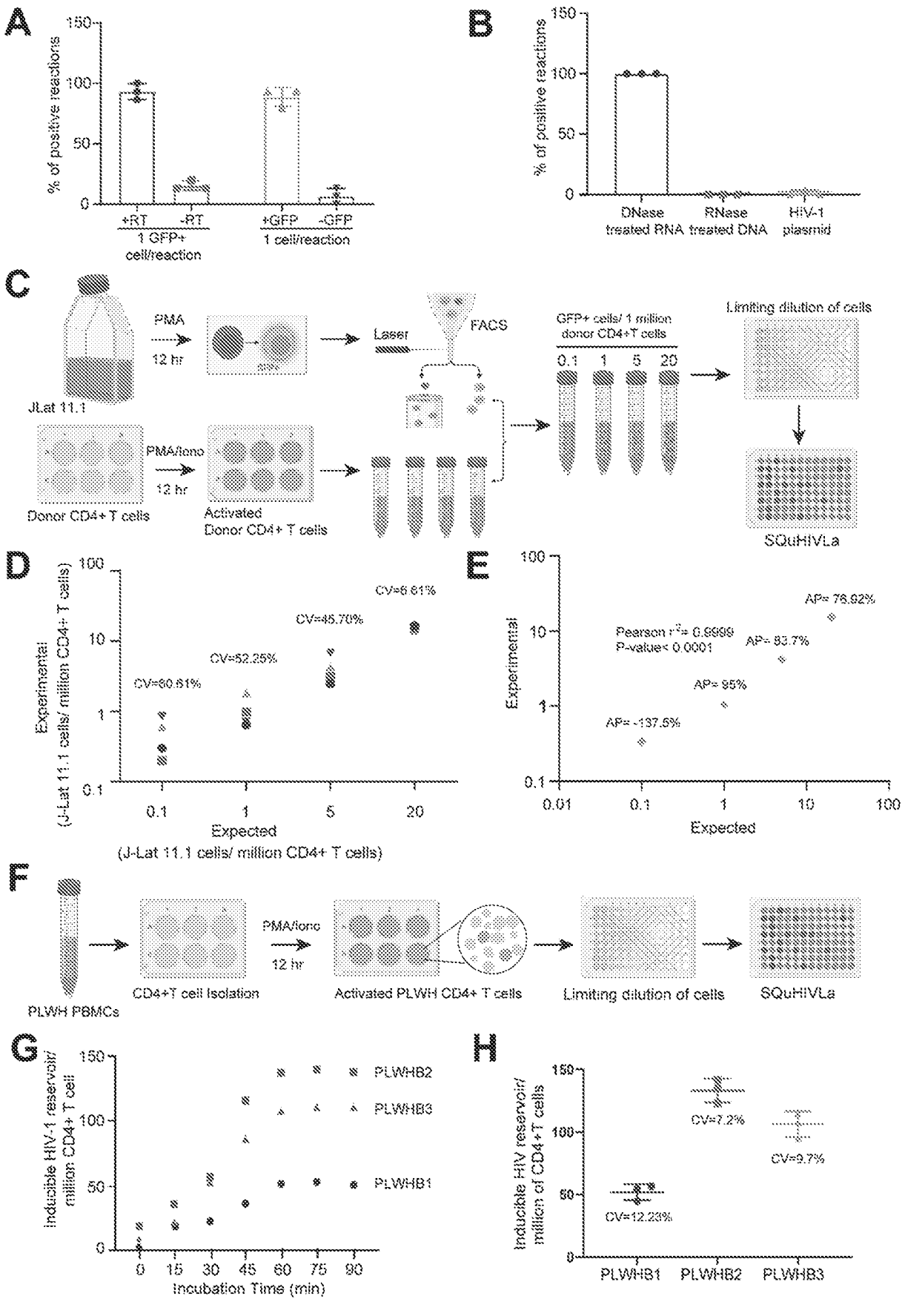
Fig. 3



Experiments	Total reactions	Positive reactions
Experiment 1	24	23
Experiment 2	24	22
Experiment 3	24	23
Experiment 4	24	24

Fig. 4


A
% of positive reactions
150
100
50
0
+RT
-RT
1 GFP+ cell/reaction
+GFP
-GFP
1 cell/reaction
B
% of positive reactions
150
100
50
0
DNase treated RNA
RNase treated DNA
HIV-1 plasmid
C
JLat 11.1
PMA
12 hr
Laser
FACS
GFP+ cells/ 1 million donor CD4+T cells
0.1
1
5
20
Limiting dilution of cells
SQuHIVLa
Donor CD4+ T cells
PMA/Iono
12 hr
Activated Donor CD4+ T cells
D
Experimental (J-Lat 11.1 cells/ million CD4+ T cells)
100
10
1
0.1
CV=60.61%
CV=52.25%
CV=45.70%
CV=6.61%
0.1
1
5
20
Expected
(J-Lat 11.1 cells/ million CD4+ T cells)
E
Experimental
100
10
1
0.1
Pearson r²= 0.9999
P-value< 0.0001
AP= 76.92%
AP= 83.7%
AP= 95%
AP= -137.5%
0.01
0.1
1
10
100
Expected
F
PLWH PBMCs
CD4+T cell Isolation
PMA/Iono
12 hr
Activated PLWH CD4+ T cells
Limiting dilution of cells
SQuHIVLa
G
Inducible HIV-1 reservoir/ million CD4+ T cell
150
100
50
0
PLWHB2
PLWHB3
PLWHB1
0
15
30
45
60
75
90
Incubation Time (min)
H
Inducible HIV reservoir/ million of CD4+T cells
150
100
50
0
CV=7.2%
CV=9.7%
CV=12.23%
PLWHB1
PLWHB2
PLWHB3

5/12

Fig. 5

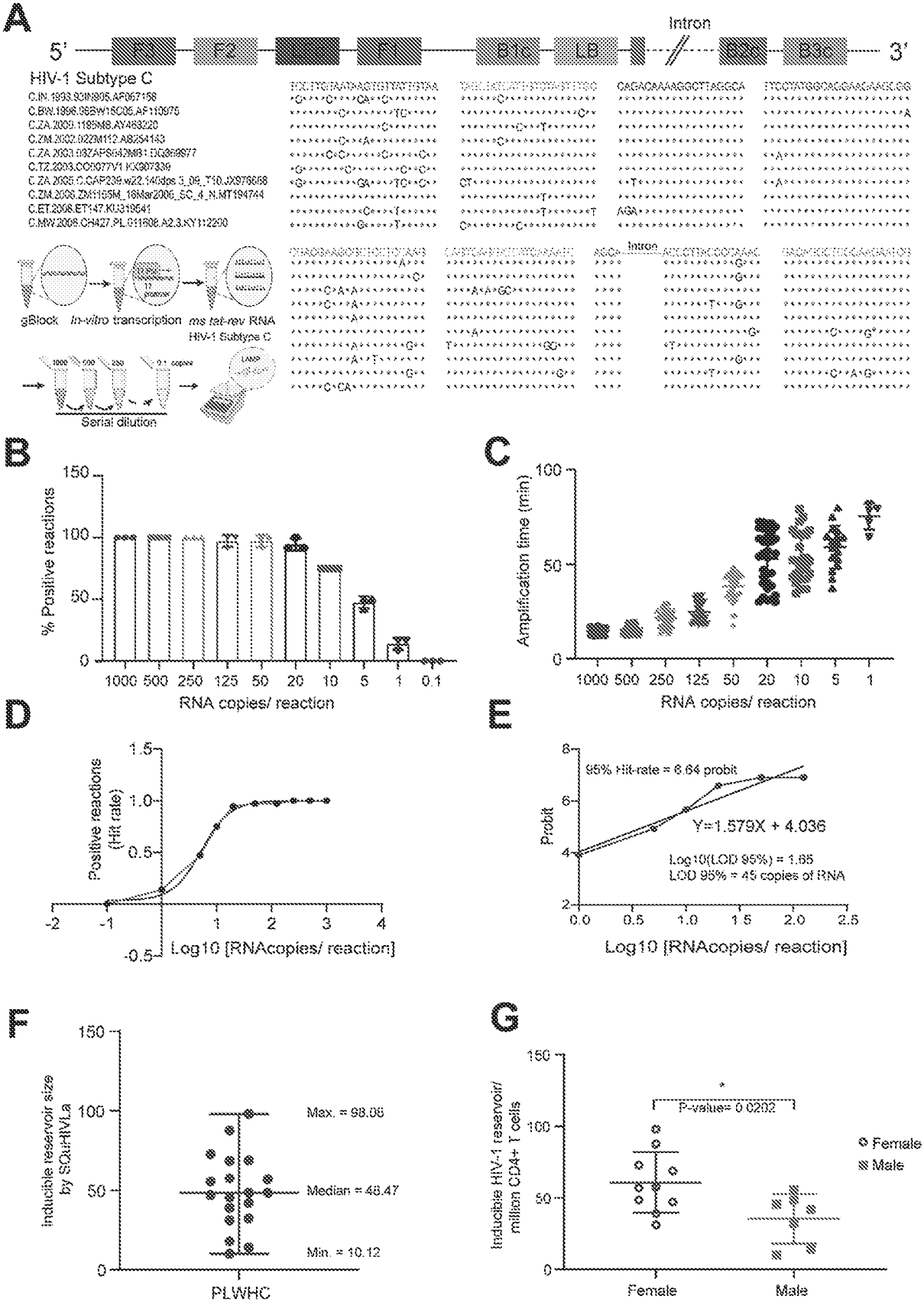

A
5'
F1
F2
C1
B1c
LB
Intron
B2c
3'
HIV-1 Subtype C
gBlock
In-vitro transcription
ms tat-rev RNA
HIV-1 Subtype C
Serial dilution
LAMP
B
% Positive reactions
RNA copies/ reaction
C
Amplification time (min)
RNA copies/ reaction
D
Positive reactions (Hit rate)
Log10 [RNAcopies/ reaction]
E
95% Hit-rate = 6.64 probit
Y=1.579X + 4.036
Log10(LOD 95%) = 1.65
LOD 95% = 45 copies of RNA
Probit
Log10 [RNAcopies/ reaction]
F
Inducible reservoir size by SCuHIVLa
Max. = 98.06
Median = 46.47
Min. = 10.12
PLWHC
G
Inducible HIV-1 reservoir/ million CD4+ T cells
P-value= 0.0202
Female
Male

Fig. 6

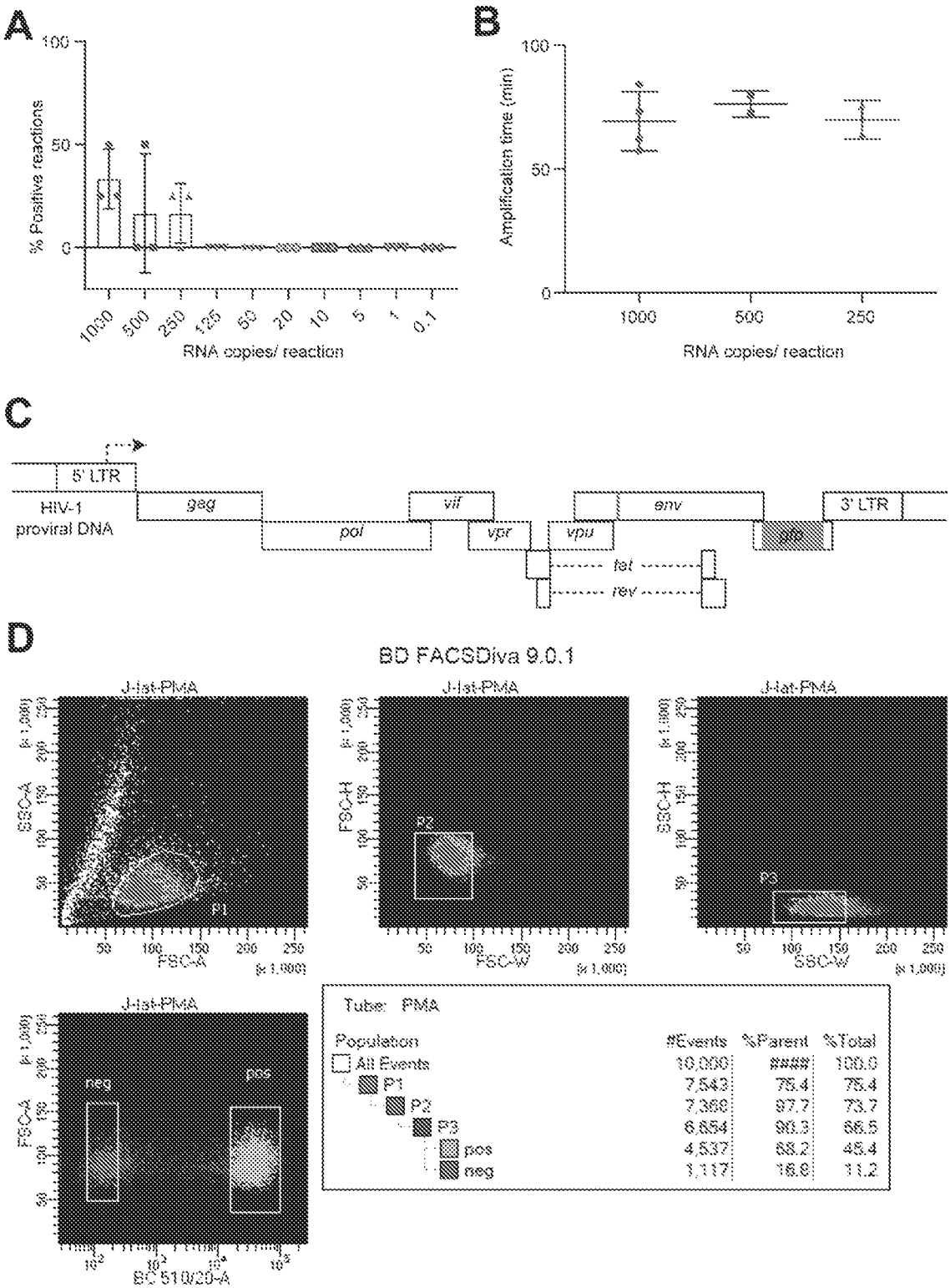
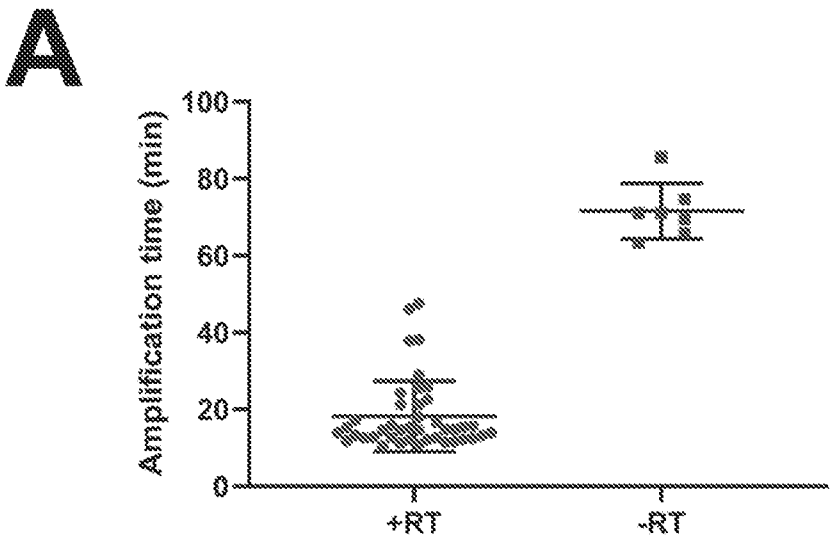
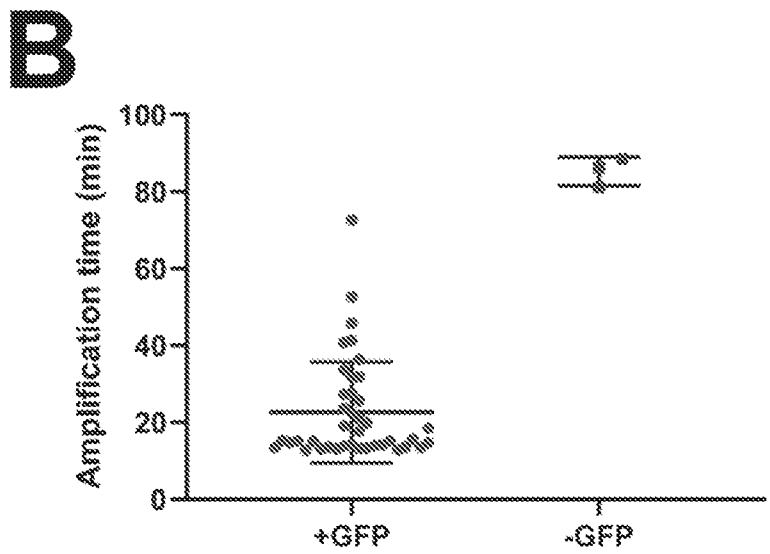

A
% Positive reactions
RNA copies/ reaction
B
Amplification time (min)
RNA copies/ reaction
C
5' LTR
HIV-1
proviral DNA
gag
pol
vif
vpr
vpu
env
3' LTR
tat
rev
D
BD FACSDiva 9.0.1
J-lat-PMA
SSC-A
FSC-A
FSC-H
SSC-H
SSC-W
P1
P2
P3
neg
pos
BC 510/20-A
Tube: PMA
Population
#Events
%Parent
%Total
All Events 10,000 #### 100.0
P1 7,543 75.4 75.4
P2 7,369 97.7 73.7
P3 6,854 93.3 68.5
pos 4,537 66.2 45.4
neg 1,117 16.8 11.2

Fig. 7


A
Amplification time (min)
100
80
60
40
20
0
+RT
-RT


B
Amplification time (min)
100
80
60
40
20
0
+GFP
-GFP

8/12
Fig. 8

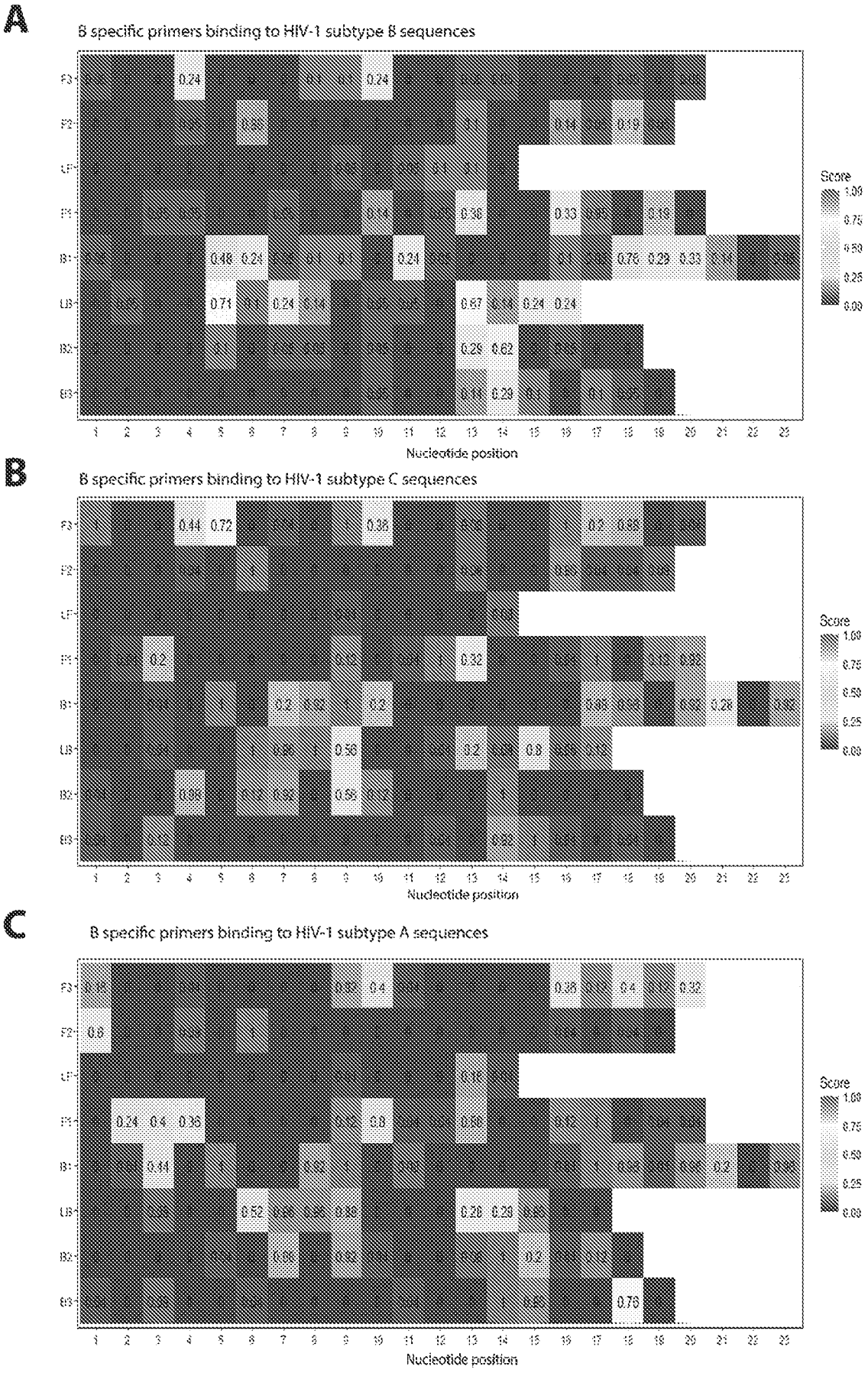
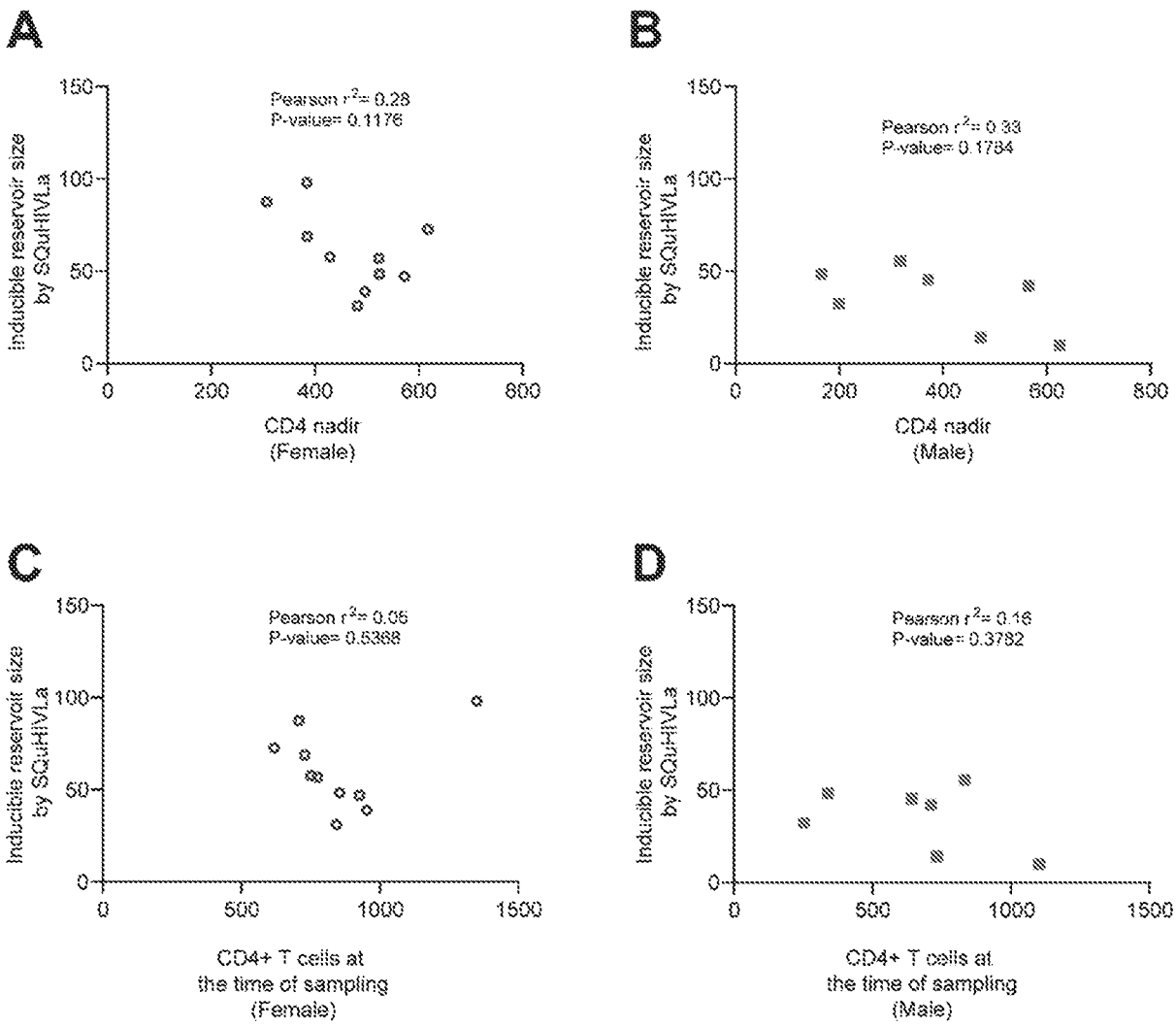

A
B specific primers binding to HIV-1 subtype B sequences
B
B specific primers binding to HIV-1 subtype C sequences
C
B specific primers binding to HIV-1 subtype A sequences
Score
1.00
0.75
0.50
0.25
0.00
Nucleotide position

Fig. 9


A
Pearson r²= 0.28
P-value= 0.1176
Inducible reservoir size by SQuHIVLa
CD4 nadir (Female)
B
Pearson r²= 0.33
P-value= 0.1784
Inducible reservoir size by SQuHIVLa
CD4 nadir (Male)
C
Pearson r²= 0.05
P-value= 0.5368
Inducible reservoir size by SQuHIVLa
CD4+ T cells at the time of sampling (Female)
D
Pearson r²= 0.16
P-value= 0.3782
Inducible reservoir size by SQuHIVLa
CD4+ T cells at the time of sampling (Male)

5′

ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCT**A**AGACTGCTTGTACCAAT
TGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGG
AAGAAGCGGAGACAGCGACGAAGAGCTCCTCAGGACAGTCAGA*CTCATCAAGTTTCTCTATCAAAGCA*
(215th "splice site")
*ACCCACCTCCCAGCCCCGAGGGGAC*CCGACAGGCCCGAAGGAATCGAAGAAGAAGGTGGAGAGAGAGACAG
*AGACAGATCCGGTCGATTA*GTGAATGGATTCTTAGCACTTATCTGGGACGACCTGCGGAGCCTGTGCCTCTTCA
GCTACCACCGCTTG**A**GAGACTTACTCTTGATTGTAACGAGGATTGTGGAACTTCTGGGACGCAGGGGGTGGG
AAGCCCTCAAATATTGGTGGAATCTCCTACAGTATTGGAGTCAGGAACTAAAGAATAG 3′

Figure 10

5′

ATGGAGCCAGTAGATCCTAACCTAGAGCCCTGGAACCATCCAGGAAGTCAGCCT**A**AAACTCCTTGTAATAAGT
GTTATTGTAAAAAATGTAGCTATCATTGTCTAGTTTGCTTTCAGACAAAAGGCTTAGGCATTTCCTATGGCAGG
AAGAAGCGGAGACAGCGACGAAGCGCTCCTCCAAGCAGTGAGG*ATCATCAAAATCTTATATCAAAGCA*
(215th "splice site")
*ACCCTTACCCCAAACCCGAGGGGAC*CCGACAGGCTCGGAAGAATCGAAGAAGAAGGTGGAGAGCAAGACAG
*AGACAGATCCATTCGATTA*GTGAACGGATTCTTAGCACTTGCCTGGGACGACCTGCGGAGCCTGTGCCTTTTCA
GCTACCACCGATTG**A**GAGACTTCATATTGGTTGCAGCGAGAGCGGTGGAACTTCTGGGACGCAGCAGTCTCA
GGGGACTACAGAGGGGGTGGGAAGCCCTTAA

Figure 11

5′

ATGGAGCCAGTAGATCCTAACCTAGAGCCCTGGAACCATCCGGGAAGTCAGCCT**A**CAACTCCTTGTAGCAAG
TGTTACTGTAAAAAGTGTTGCTATCATTGCCAAGTTTGCTTTCTGAACAAAGGCTTAGGCATCTCCTATGGCAG
GAAGAAGCGGAGACAGCGACGAGGAACTCCTCAGAGCAGTAAGG*ATCATCAAAATCCTATACCAAAGCA*
(215th "splice site")
ACCCATACCCCAAACCCAGGGGGTCTCGACAGGCCCGGAAGAATCGAAGAAGAAGGTGGAGAGCAAGGCAG
*AGACAGATCGATTCGATTA*GTGAGCGGATTCTTAGCACTTGCCTGGGACGATCTGCGGAGCCTGTGCCTCTTC
AGCTACCACCGCTTG**A**GAGACTTCATCTTGATTGCAGCGAGGACTGTGGAACTGGGACTGAGACTGGGGTG
GGAAGGCCTCAAGTATCTGTGGAATCTCCTGTTATATTGGGGTCGGGAACTAAAAATTAG 3′

Figure 12

5’

ATGGATCCAGTAGATCCTAACCTAGAGCCCTGGAACCATCCAGGAAGTCAGCCTAGGACTCCTTGTAACAAAT
GTTATTGTAAAAAGTGTTGCTATCATTGCCAAGTTTGCTTCATAACGAAAGGCTTAGGCATCTCCTATGGCAGG
AAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCGGTCAGG*CTCATCAAGATCCTCTACCAAAGCA*
(215th “splice site”)
ACCCTCCTCCCAGCCCCGAGGGGACCCGACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAGAGCAAGGCAG
*AGGCAGATCCATTCGATTG*GTGAACGGATTCTCAGCACTTATCTGGGACGATCTGCGGAACCTGTGCCTCTTCA

GCTACCACCGCTTGAGAGACTTACTCTTAATTGCAGCGAGGATTGTGGAACTTCTGGGACGCAGGGGGTGGG
AAGCCCTCAAATATCTGTGGAATCTCCTCCAGTATTGGATTCAGGAACTAAAGAATAG 3’

Figure 13

5’

ATGGAGCCGGTAGATCCTAACCTAGAGCCCTGGAATCATCCGGGAAGTCAGCCTACAACTGCTTGTAGCAAG
TGTTACTGTAAAAAATGTTGCTGGCATTGCCAACTATGCTTTCTGAAAAAAGGCTTAGGCATCTCCTATGGCAG
GAAGAAGCGGAAGCACCGACGAGGAACTCCTCAGAGCAGTAAGG*ATCATCAAAATCCTATACCAGAGCA*
(215th “splice site”)
ACCCCTACCCATCATCAGAGGGAACCCGACAGACCCGAAGGAATCGAAGAAGGAGGTGGCGAGCAAGGCAGA
*GACAGATCCGTGCGATTA*GTGAGCGGATTCTTAGCACTTGCCTGGGACGATCTACGGAGCCTGTGCCTCTTCA

GCTACCACCGCTTGAGAGACTTCATCTTGATTGCAGCGAGGACTGTGGAACTTCTGGGACGCAGCAGTCTCA
AGGGACTGAGACGGGGGTGGGAAGGCCTCAAATATCTGGGGAATCTTCTGTTATATTGGGGCCAGGAACTAA
AAATTAG 3’

Figure 14

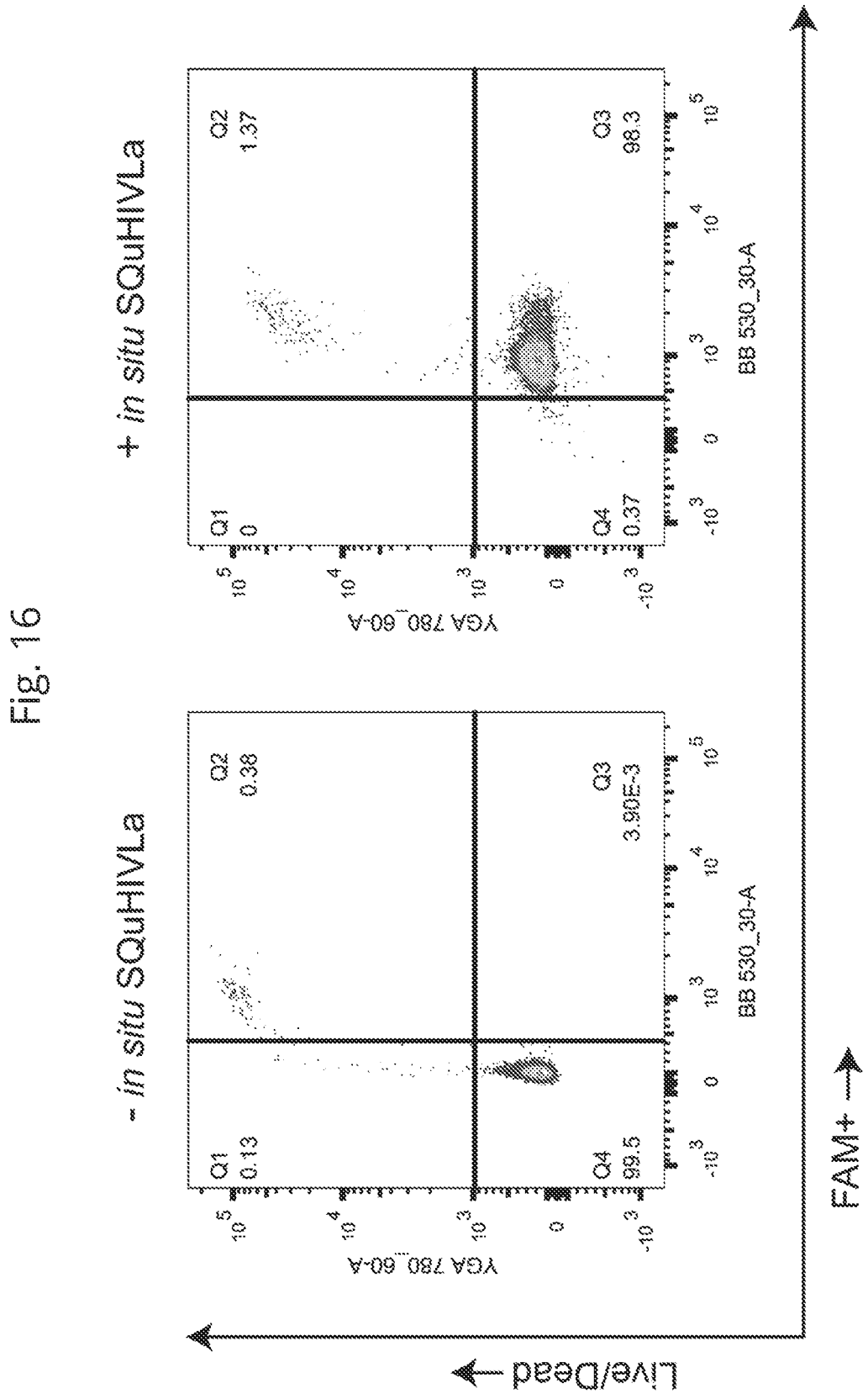
5’

ATGGAGCTGGTAGATCCTAGCCTAGAGCCCTGGAACCACCCGGGAAGTCAGCCTACAACTGCTTGTAGCAAG
TGTTACTGTAAAATGTGCTGCTGGCATTGCCAATTGTGCTTTCTGAACAAGGGCTTAGGCATCTCCTATGGCAG
GAAGAAGCGGAGACGCCGACGAGGAACTCCTCAGAGCCGTCAGG*ATCATCAAAATCCTGTACCAAAGCA*
(215th “splice site”)
ACCCTTACCCACCACCAGAGGGAACCCGACAGGCCCGAAAGAATCGAAGAAGGAGGTGGCGAGCAAGACAGA
*GACAGATCCGTGCGATTA*GTGAGCGGATTCTTAGCACTTGCCTGGGACGATCTGCGGAGCCTGTGCCTCTTCA

GCTACCACCGATTGAGAGACTTTGTCTTGATTGCTGGGACACAGCAGTCTCAAGGGCCTGAGACTGGGGTGG
GAAGCCCTCAAATATCTGTGGAATCTTCTATCATACTGGGGTCAGGAACTAAAGAATAG 3’

Figure 15

Fig. 16


- in situ SQuHIVLa
+ in situ SQuHIVLa
Q1
0.13
Q2
0.38
Q3
3.90E-3
Q4
99.5
Q1
0
Q2
1.37
Q3
98.3
Q4
0.37
YGA 780_60-A
BB 530_30-A
Live/Dead
FAM+

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2024/050359

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/70
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCOPIO FRANCESCO ANDREA ET AL: "A Novel Assay to Measure the Magnitude of the Inducible Viral Reservoir in HIV-infected Individuals", EBIOMEDICINE , vol. 2, no. 8 1 August 2015 (2015-08-01), pages 874-883, XP093108011, NL ISSN: 2352-3964, DOI: 10.1016/j.ebiom.2015.06.019 Retrieved from the Internet: URL:https://www.thelancet.com/action/showPdf?pii=S2352-3964(15)30048-7 p. 876-877, para. 2.3-2.4; p. 877, para. 3.2; p. 882, right-hand col., 1st para. ----- -/--	1-16

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 2024	09/10/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Ripaud, Leslie

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YASUYOSHI MORI ET AL: "Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases", JOURNAL OF INFECTION AND CHEMOTHERAPY ; OFFICIAL JOURNAL OF THE JAPANESE SOCIETY OF CHEMOTHERAPY AND THE JAPANESE ASSOCIATION FORINFECTIOUS DISEASES, SPRINGER-VERLAG, TO, vol. 15, no. 2, 25 April 2009 (2009-04-25) , pages 62-69, XP019664186, ISSN: 1437-7780 abstract; p. 63; fig. 1; p. 65, right-hand col., last para.	1-16
Y	SU FENGXIA ET AL: "Real-time detection of mRNA splicing variants with specifically designed reverse-transcription loop-mediated isothermal amplification", RSC ADVANCES , vol. 10, no. 11 10 February 2020 (2020-02-10), pages 6271-6276, XP093108623, GB ISSN: 2046-2069, DOI: 10.1039/D0RA00591F Retrieved from the Internet: URL:https://pubs.rsc.org/en/content/articlepdf/2020/ra/d0ra00591f abstract; p. 6273, para. bridging left- to right-hand col.; fig. 1	1-16
Y	BERTOLDI ALESSIA ET AL: "Development of C-TILDA: A modified TILDA method for reservoir quantification in long term treated patients infected with subtype C HIV-1", JOURNAL OF VIROLOGICAL METHODS, ELSEVIER BV, NL, vol. 276, 19 November 2019 (2019-11-19), XP086038877, ISSN: 0166-0934, DOI: 10.1016/J.JVIROMET.2019.113778 [retrieved on 2019-11-19] abstract; p. 2-3, para. 2.4	1-16
A	US 2019/046985 A1 (KANG DONG-KU [US] ET AL) 14 February 2019 (2019-02-14) para. 67, 100, 102	1-16
A	US 2022/127670 A1 (ZHOU CAN [US] ET AL) 28 April 2022 (2022-04-28) the whole document	1-16

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INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2024/050359

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CURTIS K A ET AL: "Rapid detection of HIV-1 by reverse-transcription, loop-mediated isothermal amplification (RT-LAMP)", JOURNAL OF VIROLOGICAL METHODS, ELSEVIER BV, NL, vol. 151, no. 2, 1 August 2008 (2008-08-01), pages 264-270, XP022832559, ISSN: 0166-0934, DOI: 10.1016/J.JVIROMET.2008.04.011 [retrieved on 2008-06-03] the whole document	1-16
A	GADKAR VIJAY J. ET AL: "Real-time Detection and Monitoring of Loop Mediated Amplification (LAMP) Reaction Using Self-quenching and De-quenching Fluorogenic Probes", SCIENTIFIC REPORTS , vol. 8, no. 1 3 April 2018 (2018-04-03), XP093108334, US ISSN: 2045-2322, DOI: 10.1038/s41598-018-23930-1 Retrieved from the Internet: URL:https://www.nature.com/articles/s41598-018-23930-1.pdf the whole document	1-16
A	Anonymous: "Advanced primer design - PrimerExplorer V5", , 31 August 2019 (2019-08-31), XP093108349, Retrieved from the Internet: URL:https://primerexplorer.jp/e/v5_manual/pdf/PrimerExplorerV5_Manual_3.pdf [retrieved on 2023-12-04] the whole document	1-16
X,P	Hossain Tanvir ET AL: "SQuHIVLa: A novel assay for Specific Quantification of inducible HIV-1 reservoir by LAMP", bioRxiv, 14 July 2023 (2023-07-14), XP093108018, DOI: 10.1101/2023.07.14.548928 Retrieved from the Internet: URL:https://www.biorxiv.org/content/10.1101/2023.07.14.548928v1.full.pdf [retrieved on 2023-12-01] the whole document	1-16

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL2024/050359

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [ ] forming part of the international application as filed.

   b. [X] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [X] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2024/050359

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2019046985 A1	14-02-2019	US 2019046985 A1	14-02-2019
		WO 2017048975 A1	23-03-2017
US 2022127670 A1	28-04-2022	NONE	