HIV POC testing by ssDNA coupled with NALF

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INTRODUCTION

HIV viral load (VL) testing is used for monitoring treatment, determining prognosis and risk of disease progression and determining treatment failure[1]. Current laboratory based VL assays rely on automated high throughput screening of samples with amplification technologies based on real-time, target or signal amplification. A rapid point of care (POC) test based on nucleic acid technology (NAT) for early and rapid detection of HIV-1 RNA in seronegative individuals, although currently unavailable, would have a significant impact on HIV diagnosis and monitoring.

Advances in the field of molecular diagnosis have seen the development of isothermal amplification techniques for detection of nucleic acids. The term 'isothermal' indicates that the entire amplification process is performed at one uniform temperature. As no temperature cycling is required, the entire reaction can be performed on a simple heating block, making it a more suitable, less expensive and easier option for nucleic acid amplification at POC.

We describe the development of a novel isothermal amplification method termed Reverse Transcription Loop Dependant Amplification (RT-LDA) which is designed to amplify HIV *gag* RNA and generate ssDNA amplicons. The design of the RT-LDA is based on strand displacement amplification (SDA)[2]. The uniqueness of the RT-LDA reaction is achieved though a primer design with a *Bso*BI recognition site that nicks ssDNA and initiates a series of strand displacement/polymerisation steps driven by *Bst* polymerase. This ssDNA product can then be detected by an inexpensive and simple detection system such as nucleic acid lateral flow (NALF) technology. NALF makes use of nucleic acid hybridization for the capture and detection of ss amplicons, similar to lateral flow immunoassays.

MATERIALS AND METHODS

Synthesis of RNA transcript

The RT-LDA technique was optimised and evaluated using *in vitro* transcribed RNA containing 128 bases of HIV-1 *gag* gene which was synthesized by Geneart (Regensburg, Germany).

Novel Primer design - Loop Mediating Primer (LMP)

The design includes an HIV specific forward (F) primer with a 5' overhang containing an artificial sequence (black), a *Bso*BI recognition site (red), a sequence complementary to the amplicon, which will form the loop (blue), and a short linking sequence between the loop and the overhang (green).

5' ——— 3'

The reverse primer (R) comprises of three regions, at the 5' end there is an artificial (random) sequence (black), a *Bso*BI recognition site (black) and HIV-1 sequence specific (reverse) primer (blue).

5' ——— 3'

RT-LDA reaction mater mix

Total volume of 50 µl = 20 µl of template RNA and 30 µl of reaction mix. The reaction mix was assembled as follows: 35 mM Potassium phosphate buffer (K_1PO_4), 5% v/v dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA), 4% v/v glycerol (Promega, Madison, WI, USA), 5 µg acetylated bovine serum albumin (AcBSA; Promega, Madison, WI), 0.8 mM 2'-deoxycytidine 5'-O- (1- thiotriphosphate) (dCTP - αS; Amersham Biosciences, Piscataway, NJ, USA), 0.6 mM dUTP, 0.2 mM each dATP and dGTP (Promega, Madison, WI, USA), 7.5 mM magnesium acetate ($MgOAc_2$; Sigma-Aldrich, USA), 7.5 units AMV reverse transcriptase (USB Corp., Cleveland, Ohio, USA), 32 units *Bso*BI restriction enzyme (New England BioLabs Inc., USA), 20 units *Bst* DNA polymerase (New England BioLabs Inc., USA), 1µg T4gp32 ssDNA binding protein (USB Corp., Cleveland, Ohio, USA), forward and reverse primers 0.75 µM each.

Incubation at 53 ºC for 1 hour on a heating block (Labcon, South Africa).

Detection of RT-LDA amplicon

Nucleic Acid Lateral Flow (NALF)

All reagents, including buffers, magnetic conjugate, nitrocellulose DNP striped half dipsticks, capture and detection probes were manufactured by British Biocell International (Cardiff, UK). Sequences of the lateral flow probes:

Ampi 2032 = Detection probe (two inosines):
5' Biotin TCTGC ACA TCC AGI ICA TGC GGG – 3'
Ampi 2033 = DNP – capture probe (two inosines):
5' – GCC TII TGC ACC AGG CCT – DNP- 3'
Ampi 29 = positive control (a synthetic ssDNA molecule that has a partially looped 'structure' and a sequence similar to the real amplification product).

Autoradiography

RT-LDA: 0.4mM [α-^{32}P]dATP (IZOTOP,AEC Amersham) was incorporated into the RT-LDA reaction to radiolabel the ssDNA amplicons.

Clean-up: the RT-LDA reaction was purified with a phenol /chloroform ethanol extraction.

PAGE: The product was resolved by running on a denaturing 15% Acrylamide/8M Urea gel for approximately 2 hours at 150V. The gel was fixed in a 10% acetic acid: 10% methanol solution for two hours and dried using a gel drier system. Gels were then exposed to X-ray film overnight before being developed.

RESULTS

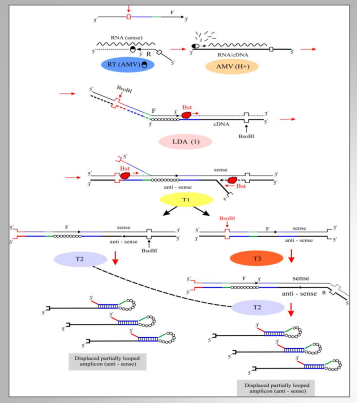

Figure 1: Design of RT-LDA. The RT-LDA reaction proceeds as follows: *Linear phase* - the reverse primer (R) binds viral RNA and gets extended by the AMV reverse transcriptase (RT) to form a ds RNA/cDNA hybrid. RNase H activity (AMV H+), of AMV RT digests the viral RNA in the hybrid leaving only the cDNA (anti-sense) sequence. The LMP (F) binds to the cDNA (anti-sense) strand and is extended by *Bst* polymerase resulting in a dsDNA target (T1). Strands complementary to the *Bso*BI restriction site are generated during the primer/strand extension but due to the addition of chemically modified dCTP (dCTP - αS) in the reaction mix , the double stranded *Bso*BI recognition site is hemithiolated. This prevents *Bso*BI from cutting the dsDNA and instead, only the unmodified nickable sites (in all targets - T1, T2 and T3) from the primers are nicked (ss nick) by the enzyme. T1 dsDNA has two nickable sites – one on the sense strand from the forward primer (red box) and another on the anti-sense strand from the reverse primer (black box). Both strands are recognized and cleaved by *Bso*BI so that the 3'- end of each nicked strand (sense and anti –sense) can be extended by *Bst* DNA polymerase whilst the existing strands are displaced. The displaced sense strand forms a amplicon which has a sequence complementary to the reverse primer resulting in the reverse primer binding to it and converting it into dsDNA target (T2). T2 will have only one *Bso*BI restriction site, and a process on nicking and strand displacement/ polymerization occurs on the T2 target in a similar way as described above. This cascade of processes between formations of T1 and T2, represent the linear phase of RT-LDA. ***Cycling phase*** - The displaced anti-sense strand from T1, which possesses a 3' sequence complementary to the forward primer, binds the forward primer and is converted to a dsDNA target (T3). Extension of T3 produces "looped" sense amplicons similarly to T1 which will bind to the reverse primer and lead to the formation of dsDNA targets identical to T2. The cascade of processes, from formation of T1 to T3 and then to T2, represents the semi-cycling phase of RT-LDA. T2 generated during the semi-cycling phase also keeps producing "looped" ssDNA anti-sense amplicons.

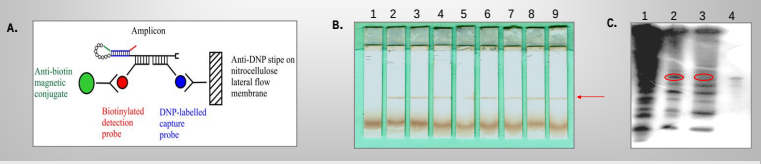

Figure 2: A.) Design of lateral flow dipsticks and superparamagnetic conjugates B.) Detection of RT-LDA amplicons (arrow) using NALF dipsticks. From left to right: RT-LDA blank sample (1); two positive controls containing an artificially synthesized looped amplicon (ampi29) (2, 3); serial dilutions of RT-LDA amplicon products (4-9). C.) Autoradiograph of PAGE gel following RT-LDA reaction and phenol/chloroform ethanol precipitation. Lane 1: non-extracted control showing smear; Lane 2: RT-LDA extracted sample showing 173pb amplicon; Lane 3: extracted water blank showing bands of various sizes but the correct sized amplicon is absent; Lane 4: RT-PCR control amplicon of 173bp for product size verification.

CONCLUSION

- RT-LDA rapidly converts HIV *gag* RNA template into ssDNA amplicons within 1 hour in an isothermal amplification using a simple heating block.
- The ssDNA product can be detected by lateral flow (NALF) dipsticks over a wide range of template concentrations ($4x10^3$ to $4x10^8$ copies/ml).
- Once combined with a rapid extraction protocol the RT-LDA combined with NALF may be a suitable option for HIV POC testing and monitoring.

LITERATURE CITED

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