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# Strategies to quantify unspliced and multiply spliced mRNA expression in HIV-2 infection

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## ABSTRACT

HIV-2 infection is associated with a slower rate of disease progression with limited impact on the survival of the majority of infected adults, and much lower plasma viral load than HIV-1. In spite of the major differences in viremia, the quantitative assessment of HIV-2 proviral load documented levels similar to those observed in HIV-1 infected individuals, suggesting an equivalent number of circulating infected cells in both infections. It remains unclear whether this apparent paradox results from a contribution of latent/quiescent viruses or from transcriptional and/or post-transcriptional control of HIV-2 replication. In order to investigate these possibilities, a one-step and two-step reverse transcription quantitative real-time PCR based methods (RT-qPCR) for *gag* and *tat* mRNA HIV-2 transcripts were developed. These methods were validated and compared to assess the expression of HIV-2 *gag* and *tat* transcripts in parallel with proviral DNA and viral production. The results suggest that the two-step approach may allow a better detection of low level *gag* and *tat* mRNA HIV-2 transcripts.

## 1. Introduction

HIV-2 infection is characterized by levels of viremia much lower than those of HIV-1 (Andersson et al., 2000; Berry et al., 1998; Popper et al., 1999; Simon et al., 1993; Soriano et al., 2000). However, the levels of cell-associated DNA-provirus have been shown to be similar in the two infections suggesting that the number of cells harboring viral DNA does not differ significantly (Berry et al., 1994; Gomes et al., 1999; Popper et al., 2000; Soares et al., 2006). Thus, HIV-2 seems to be able to disseminate and establish a reservoir of infected cells similarly to HIV-1. It remains unclear whether this HIV-2 reservoir represents mainly cells infected with latent HIV or if a significant degree of HIV-2 replication is occurring and the production of new virions is controlled at the post-transcriptional level. Several mechanisms have been suggested to contribute to this putative control of viral replication including HIV-2 specific immune responses (Duvall et al., 2006; Foxall et al., 2008), that were thought to contribute to the relatively benign course of HIV-2 infection (Drylewicz et al., 2008; Marlink et al., 1994).

HIV genes are expressed through complex splicing of a single mRNA precursor, generating more than 40 distinct mRNA species that can be grouped into three classes: unspliced (US) full length 9-kb mRNA, partially spliced 4–5 kb mRNA, and multiply spliced (MS) ~2-kb mRNA (Neumann et al., 1994; Schwartz et al., 1990, 1992). US mRNA encodes the Gag and Gag-Pol polyproteins and also serves as the genomic viral RNA. MS mRNA encodes the regulatory HIV proteins Tat, Rev and Nef.

Kinetic analysis of HIV-1 mRNA expression *in vitro* in newly infected cells showed an early expression of mostly MS mRNA followed by a subsequent increase in the levels of US mRNA (Kim et al., 1989; Klotman et al., 1991; Ranki et al., 1994), suggesting that a large proportion of cells expressing HIV-1 MS mRNA are thought to have been recently infected (Bagnarelli et al., 1996).

Data on HIV-2 transcription are scarce. There is only one report addressing the expression of unspliced mRNA in patients infected with HIV-2 (MacNeil et al., 2007), namely *gag* transcripts. Furthermore, there are no data on the quantitation of HIV-2 multiply spliced viral mRNA, which highlights the need for further studies to clarify if the low viremia observed in the majority of patients infected with HIV-2 is associated with an effective control of viral transcription. Since patients infected with HIV-2 usually exhibit low to undetectable viremia, it is expected that the levels of viral transcripts might be low, highlighting the need to develop sensitive assays for the quantitation of HIV-2 transcripts.

Two-step RT-qPCR has been the most frequently used method for the quantitation of both unspliced and multiply spliced viral mRNA in HIV-1 infection (Brachtel et al., 2002; Furtado et al., 1995; Hermankova et al., 2003; Mackewicz et al., 2000; Pasternak et al., 2008, 2009) and unspliced viral mRNA in patients infected with HIV-2 (MacNeil et al., 2007). Recently, approaches based on one-step RT-qPCR have been developed that may have some advantages

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over two-step RT-qPCR (Wacker and Godard, 2005). In the latter approach the reverse transcription and the real-time PCR assay are performed in separate reactions. However, it is known that most of the variability in RT-qPCR is mainly attributable to the reverse transcription step, which has relatively high variability in its reaction efficiency (Stahlberg et al., 2004), and to errors associated with successive pipetting steps during the preparation of the reactions. These factors may pose a problem for quantitation, particularly when transcripts are present at low copy numbers, and/or if absolute quantitation of mRNA is required. The one-step RT-qPCR approach is thought to minimize experimental variation since both enzymatic reactions occur in the same tube, and it involves less sample-handling, reducing the chance of contamination and/or pipetting-associated errors. Overall, these differences make this method more suitable for absolute quantitation. The protocol can be completed in a shorter time-period, thus minimizing sample-degradation. There are so far no studies comparing these two methods in the quantitation of HIV-1 and HIV-2 viral transcripts.

Quantitation of HIV-2 transcripts requires the development of particularly sensitive assays given the low to undetectable viremia usually observed in patients infected with HIV-2. One-step RT-qPCR has been suggested to be less sensitive than two-step protocols (Battaglia et al., 2009). However, a more detailed study comparing the efficiency and sensitivity of the two approaches for the quantitation of high, intermediate and low transcript expression suggests that one-step RT-qPCR has a higher sensitivity for the detection of low transcript levels, and that the two methods have a similar reaction efficiency (Wacker and Godard, 2005). Other factors are known to impact on the efficiency and sensitivity of RT-qPCR, namely the priming strategy and the concentration of RNA used in the reverse transcription step (Lekanne Deprez et al., 2002; Stahlberg et al., 2004), as well as the quantity of cDNA used in the qPCR (Lekanne Deprez et al., 2002). Thus, further development and comparison of both approaches are required in order to develop sensitive assays for the quantitation of HIV-2 transcripts.

This study reports the development and validation of one-step and two-step RT-qPCR methods for the analysis of HIV-2 unspliced and multiply spliced mRNA expression. The ability of these methods to analyze HIV-2 *gag* and *tat* mRNA expression upon *in vitro* infection was compared.

## 2. Materials and methods

### 2.1. Samples used and in vitro infections

Peripheral blood mononuclear cells (PBMCs) isolated from leucocyte-enriched buffy coats from healthy blood donors (Instituto Português de Sangue, Lisbon) by Ficoll-Hypaque (Amersham Pharmacia Biotech, Little Chalfont, UK) gradient centrifugation were used for *in vitro* infections, under the approval of the Ethical Board of the Faculty of Medicine, University of Lisbon. Pooled PBMCs from three different donors were stimulated with phytohemagglutinin for 48 h (PHA; 3 μg/ml; Sigma–Aldrich, St. Louis, MO, USA) and infected subsequently with either HIV-2 ROD (MRC, UK) or HIV-2 primary isolates (Table 1, all viruses, except 20.04, were obtained from AIDS Reagent Program, National Institutes of Health, USA. HIV-2 20.04 was kindly provided by Nuno Taveira from Faculdade de Farmácia da Universidade de Lisboa). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (all from Gibco-Invitrogen, Paisley, UK). PBMCs were infected using medium supplemented with 10 UI/ml of human recombinant IL-2, and 5 μg/ml of polybrene (Sigma–Aldrich, St. Louis, MO, USA) for 4 h. Non-infected PBMCs were used as negative controls for the one-step and two-step qPCR assays. Positive controls for these assays were generated from H9 cells ($2 \times 10^6$ cells/ml) infected in the presence of polybrene (5 μg/ml) with cell-free culture supernatants containing the reference viral strains HIV-2 ROD or HIV-1 IIIB (AIDS Reagent Program, National Institutes of Health, USA) at more than 0.3 ng p24 equivalents/ml, and cultured for 48 h upon infection. These H9 infected cells were also used to evaluate the specificity and sensitivity of the one-step and two-step qPCR assays.

**Table 1**
Characteristics of the HIV-2 strains.

| Viral strain | LA/PI[a] | Coreceptor usage |
|---|---|---|
| ROD[b] | LA | CCR5/CXCR4 |
| 60415K[c] | PI | CCR5 |
| 20.04[d] | PI | CXCR4 |
| CDC310342[c] | PI | MULTI[e] |

[a] LA, lab adapted; PI, primary isolate.
[b] From MRC, UK.
[c] From AIDS Reagent Program, NIH, USA.
[d] Kindly provided by Nuno Taveira.
[e] Multi, multiple coreceptor usage: CCR5, CXCR4, CCR1, STRL33.

To evaluate the ability of each assay to quantify HIV-2 transcripts at different time points after infection, pooled PBMCs were infected with 0.125 ng reverse transcriptase (RT) per $3 \times 10^6$ PBMCs of HIV-2 ROD, cultured in medium supplemented with IL-2 (10 UI/ml). Culture supernatants and cell pellets were collected at 24, 72, 94 and 120 h after infection. *gag* and *tat* mRNA, and RT activity were quantified at all time-points, with total proviral DNA quantitation performed on the final sample (120 h after infection).

To evaluate the efficiency of these assays in detecting different viral strains, HIV-2 primary isolates utilizing either single CCR5 (60415 K), CXCR4 (20.04) or multiple CCR5, CXCR4, CCR1 and STRL33 (CDC310342) co-receptors (Table 1) were used to infect PBMCs at 0.5 ng RT/$3 \times 10^6$ cells. Cells and culture supernatants were collected 72 h post-infection for quantitation of *gag* and *tat* mRNA, and RT activity respectively; and at 120 h for proviral DNA quantitation.

### 2.2. Isolation of total proviral DNA, total RNA and mRNA

DNA and total RNA were extracted from pellets of $5 \times 10^6$ cells using QIAamp DNA Mini Kit and RNeasy mini kit respectively (Qiagen, Valencia, CA), according to the manufacturer's instructions. For mRNA extraction, $5 \times 10^6$ cells were first lysed in 0.6 ml of Qiagen RLT buffer and were homogenized further using Qiashredder columns (Qiagen, Valencia, CA). Polyadenylated mRNA was extracted using Oligotex mRNA direct mini kit (Qiagen, Valencia, CA), treated with DNase (DNA-free kit, Ambion, Austin, TX) and converted immediately to cDNA, or used directly in one-step RT-PCR assays. DNA, mRNA and cDNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE).

### 2.3. Target sequences, primers and probes

Target nucleic acid sequences were selected in specific and conserved regions of the genome. HIV-2 *tat* primers and probe were designed to span an exon–exon splice junction in order to guarantee the amplification and detection of RNA sequences only. HIV-2 *gag* and *tat* oligonucleotide probes with a reporter 5′-6-carboxyfluorescein dye (FAM) and a 3′-Minor Groove Binder (MGB) group were used. MGB allows the formation of highly stable duplexes with single-strained DNA targets allowing shorter probes to be used, resulting in increased specificity (Kutyavin et al., 2000). For the selection of candidate primers and probes the Primer Express software package (Applied Biosystems) was used.