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Journal of Virological Methods 138 (2006) 201–206
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Short communication

PCR-based detection is unable to consistently distinguish HIV 1LTR circles

Kristine E. Yoder*, Richard Fishel

Comprehensive Cancer Center, The Ohio State University, 400 W. 12th Ave. Room 351, Columbus, OH 43210, United States

Received 2 May 2006; received in revised form 19 July 2006; accepted 25 July 2006
Available online 7 September 2006

Abstract

Quantitative PCR methods are routinely used to measure multiple HIV cDNA forms, including linear cDNA, early and late reverse transcripts, 2LTR circles, and integrated provirus. PCR-based methods for the detection of 1LTR circles have been proposed, but are complicated by the inherent homology of the LTR sequence present in all cDNA forms. Amplicons with variable lengths of homology showed that it is difficult to discriminate 1LTR circles faithfully from other cDNA forms. Addition of formamide, DMSO, or glycerol did not eliminate amplification of spurious products. Thus, detection of 1LTR circles by PCR is not reliable.
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Keywords: HIV; PCR; DNA circles

Following entry into a target cell, HIV reverse transcribes the genomic RNA to a double-stranded linear cDNA molecule (Coffin et al., 1997). The linear cDNA is part of a pre-integration complex (PIC) which enters the nucleus where it must complete integration into the host chromosome to continue the viral life cycle (Englund et al., 1995; Sakai et al., 1993). Nuclear resident host proteins may convert the linear viral cDNA to circular products containing 1LTR or 2LTRs. The circular cDNA molecules are only found in the nuclear compartment and have served as an experimental marker of PIC entry to the nucleus (Barbosa et al., 1994; Zennou et al., 2000). Quantification of circles has also been used as an indicator of continuing viral replication in HIV infected individuals treated with highly active antiretroviral therapy, although the utility of this method is controversial since the circles are stable dead end products that do not support viral replication (Fischer et al., 2003; Pierson et al., 2002; Sharkey et al., 2000).

Quantitative PCR (qPCR) methods have made the measure of HIV late reverse transcripts and 2LTR circles trivial (Butler et al., 2001). Primers for late reverse transcripts are complementary to HIV U5 sequence and the *gag* gene. This amplicon is present in all forms of HIV cDNA including linear cDNA, 1LTR circles, 2LTR circles, and integrated provirus. Primers specifically quantifying 2LTR circles amplify across the unique junction of LTRs. The forward primer is complementary to U5 sequence and the reverse primer is complementary to U3 sequence. Additional qPCR methods are able to quantify the integrated provirus by utilizing primers complementary to the HIV LTR and host Alu sequence (Brussel and Sonigo, 2003; Kumar et al., 2002; O'Doherty et al., 2002). Linker-mediated qPCR methods have been described to quantify linear HIV cDNA, which may have blunt ends or recessed 3′ hydroxyls (Pierson et al., 2002).

Several PCR-based methods have been proposed to measure 1LTR circles (Bukrinsky et al., 1992; Farnet and Haseltine, 1991; Heinzinger et al., 1994; Teo et al., 1997; Wu and Marsh, 2003). Common features of these methods include a forward primer complementary to 3′ sequence of the *nef* gene and a reverse primer complementary to the *gag* gene (Fig. 1). The resulting amplicon spans the single LTR sequence and appears to be unique to 1LTR circles based on size. In this scenario, more than 60% of the amplicon is identical to linear cDNA, 2LTR circles, and integrated provirus at the HIV LTR (Bukrinsky et al., 1992; Heinzinger et al., 1994; Teo et al., 1997; Wu and Marsh, 2003). Under normal circumstances, these other cDNA forms are present in excess to 1LTR circles (Zennou et al., 2000). Because of this, we tested the ability of PCR to faithfully distinguish 1LTR circles from other HIV cDNA forms. Additional primer sets generating longer amplicons with less homology were also

* Corresponding author. Tel.: +1 614 292 3328; fax: +1 614 688 4994.
E-mail address: yoder.176@osu.edu (K.E. Yoder).

0166-0934/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jviromet.2006.07.022

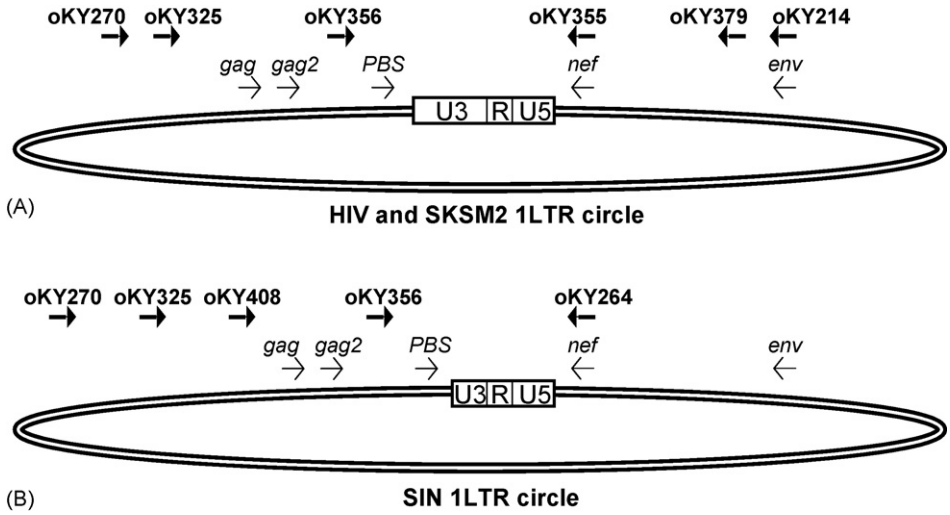

Fig. 1. Relative binding sites of PCR primers used to amplify 1LTR circles. These are schematic representations of (A) an HIV or SKSM2 retroviral vector 1LTR circle and (B) a SIN retroviral vector 1LTR circle. A deletion of 400 bp in the HIV LTR U3 yields the truncated SIN LTR U3 (Follenzi et al., 2000). The relative locations of PCR primers used in this study (bold) or previous studies (italics) are indicated. PCR primer pairs used in this study are listed in Table 1. PCR primer pairs used in previous studies include *gag* and *nef* (Heinzinger et al., 1994; Wu and Marsh, 2003), *gag2* and *nef* (Bukrinsky et al., 1992), *PBS* and *env* (Farnet and Haseltine, 1991). For simplicity, the names of PCR primers here reflect the position in the HIV sequence and are not necessarily the previously published name. Distances are relative and not to scale.

tested for their fidelity (Fig. 1, Table 1). Thus the percentage of homology was adjusted by varying the total length of the amplicon while the LTR length of either SKSM2 or SIN templates remained constant. PCR reaction conditions were as described (Wu and Marsh, 2003). Briefly, 25 μl reactions included 1× PCR Buffer with 1.5 mM $MgCl_2$, 125 μM deoxynucleotides, 1 U Taq polymerase (Roche), and 1 μM each primer (IDT). Thermal cycling began with denaturation for 2 min at 94 °C followed by 30 cycles of 94 °C 30 s, 56 °C 30 s, and 72 °C 1 min. A 10 μl aliquot from each reaction was analyzed by 1% agarose gel with ethidium bromide.

HIV-based retroviral vectors with alternative LTR sequences were produced by transfection of human fibroblast 293T cells with three plasmids: the HIV packaging construct Δ*R*9, the VSV-G envelope construct, and the genomic SKSM2 construct or SIN construct (Follenzi et al., 2000; Hansen et al., 1999). SKSM2 encodes a full length HIV_{HXB2} LTR, while 400 base pairs of the HIV_{HXB2} LTR U3 have been deleted from the SIN construct (Naldini et al., 1996; Zufferey et al., 1998). Vector supernatants were filtered and treated with DNaseI to eliminate contamination by producer plasmids (Lu et al., 2004). The 293Ts were transduced with SKSM2 or SIN. These retroviral vectors have been shown by qPCR methods to behave similarly to HIV; the time courses of reverse transcription, 2LTR circle formation, and integration are the same for both HIV infections and HIV-based vector transductions (Butler et al., 2002). Cytoplasmic PIC extracts were used as a negative control for circle amplification. PICs prepared at 5 h post-transduction contain only linear cDNA; circular cDNA molecules are found exclusively in the nucleus and typically do not form at such an early time point (Farnet and Haseltine, 1991). Briefly, the PIC extracts were generated by exposing cells to the HIV-based vector in the presence of 10 μg/ml DEAE dextran (Sigma) for 5 h (Miller et al., 1997). Cells were pelleted, washed with buffer K (20 mM HEPES (pH 7.4), 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT), lysed in buffer K with 0.05% NP-40 and protease inhibitors (leupeptin, pepstatin, and PMSF), snap frozen in liquid nitrogen, and stored at −80 °C. Total DNA was extracted (Qiagen DNeasy Tissue Kit) from uninfected cells, cells transduced for 15 h (early infected), and cells transduced and cultured for more than 3 months (late

Table 1
Retroviral vectors and primer sets

Vector	Primer pair	Amplicon length (bp)	LTR (%)
SKSM2	oKY325: GGGTTGCTACTGTATTATATAATG, oKY355: GGGACTGGAAGGGCTAATTCACTC	1061	60
SKSM2	oKY270: GCTGTGCGGTGGTCTTACTTTTG, oKY355: GGGACTGGAAGGGCTAATTCACTC	1157	55
SKSM2	oKY356: TCTCCTTCTAGCCTCCGCTAGTCA, oKY379: CAACAGCCACAACGTCTATATCAT	1307	49
SKSM2	oKY356: TCTCCTTCTAGCCTCCGCTAGTCA, oKY214: CCATCTTCTTCAAGGACGAC	1448	44
SKSM2	oKY325: GGGTTGCTACTGTATTATATAATG, oKY279: GTGAGTCAAACCGCTATCCAC	1565	41
SKSM2	oKY270: GCTGTGCGGTGGTCTTACTTTTG, oKY214: CCATCTTCTTCAAGGACGAC	1807	35
SIN	oKY356: TCTCCTTCTAGCCTCCGCTAGTCA, oKY264: AGCTGTAGATCTTAGCCACTT	424	55
SIN	oKY408: CAGCCCTTCTGATGTTTCTAACAGGCC, oKY264: AGCTGTAGATCTTAGCCACTT	599	40
SIN	oKY325: GGGTTGCTACTGTATTATATAATG, oKY264: AGCTGTAGATCTTAGCCACTT	682	35
SIN	oKY270: GCTGTGCGGTGGTCTTACTTTTG, oKY264: AGCTGTAGATCTTAGCCACTT	778	30

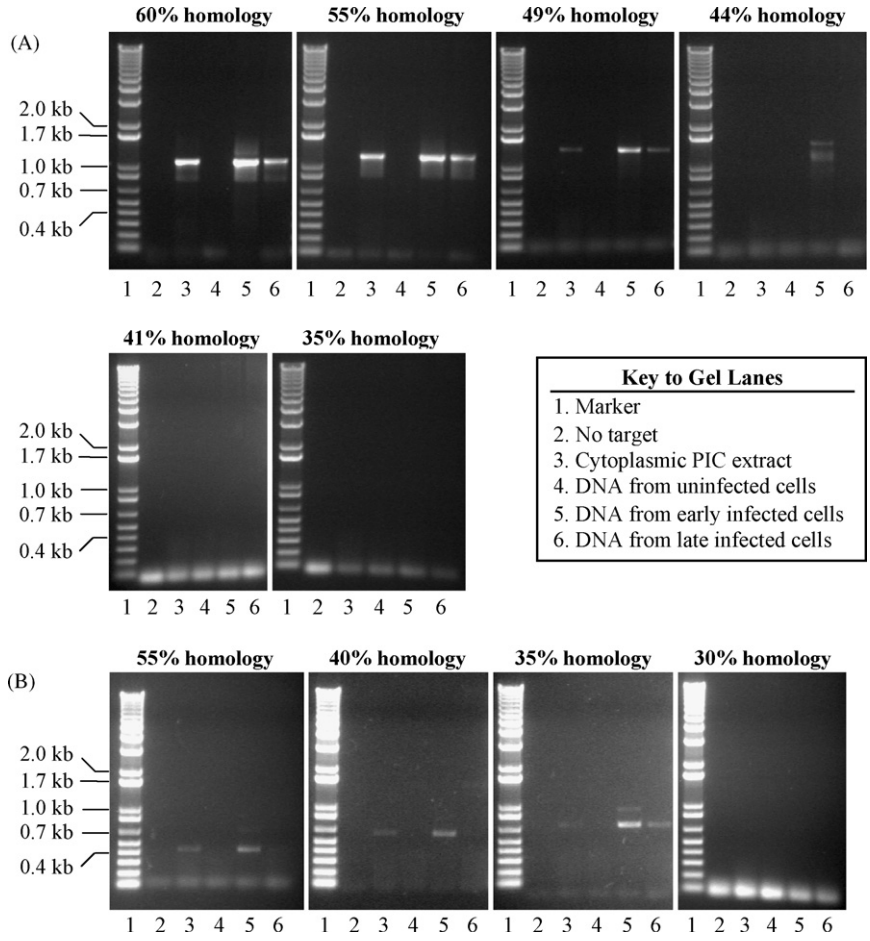

Fig. 2. PCR amplification of retroviral vector cDNA. The percentage of LTR homology in each amplicon is indicated at the top of each panel. Lanes are 1, marker; 2, no target; 3, cytoplasmic PIC extract; 4, DNA from uninfected cells; 5, DNA from early infected cells, 15 h post-addition of retroviral vector; 6, DNA from late infected cells, months following addition of retroviral vector. (A) SKSM2 retroviral vector transduced samples. (B) SIN retroviral vector transduced samples.

infected). Early infected DNA should include linear cDNA, 1LTR circles, 2LTR circles, and integrated provirus. Late infected DNA has only integrated provirus present. An equivalent amount (100 ng) of DNA was included in each amplification reaction.

When 60% of the amplicon is homologous to LTR sequence, PCR products of the predicted size for SKSM2 1LTR circles are generated from DNA of cytoplasmic PIC DNA, early infected DNA, and late infected DNA (Fig. 2A, 60% homology, lanes 3, 5, and 6). There is no amplification of uninfected cellular DNA (Fig. 2A, lane 4). Additional primer sets with decreasing LTR homology were assayed for their ability to amplify spurious products from the same target DNAs. PCR products of the predicted sizes were amplified from all HIV vector DNA samples when the LTR homology was ≥49%. The specious bands in the cytoplasmic PIC DNA and the late infected DNA disappeared only when the LTR homology was reduced to 44% of the total amplicon length (Fig. 2A, 44% homology). This primer set amplified two species from the early infected DNA (lane 5), which are the predicted sizes for 1LTR circles and 2LTR circles. When the LTR homology is further reduced to <44% of the amplicon, no amplification is detected.

These results suggested that optimization of the PCR reaction conditions may be able to faithfully amplify a 1LTR circle product. The primer set with 44% LTR homology of the amplicon appeared to give the best discrimination of 1LTR circles. However, increasing the concentration of the target DNA samples from 100 to 500 ng yielded the amplification of products of the predicted size from PIC DNA and late infected DNA (Fig. 3A). Thus an increase in target DNA quantity may obscure the accuracy of 1LTR circle amplification by these primers.

The 293T cells were also transduced with SIN, a self-inactivating HIV-based vector (Follenzi et al., 2000). The 400 base pairs of U3 have been deleted, yielding a much shorter LTR. When ≥35% of the amplicon is LTR sequence, PCR products of the predicted size for 1LTR circles are generated from PIC DNA, early infected DNA, and late infected DNA (Fig. 2B). Two bands are amplified from the early infected DNA correlating to 1LTR and 2LTR circles when the LTR homology is 35% of the amplicon (Fig. 2B, 35% homology, lane 5). No amplification was detected when the LTR homology was reduced to 30% of the amplicon length, although the reaction conditions should be able to generate a 778 base pairs (bp) product. The target DNA concentration was increased from 100 to 500 ng and the

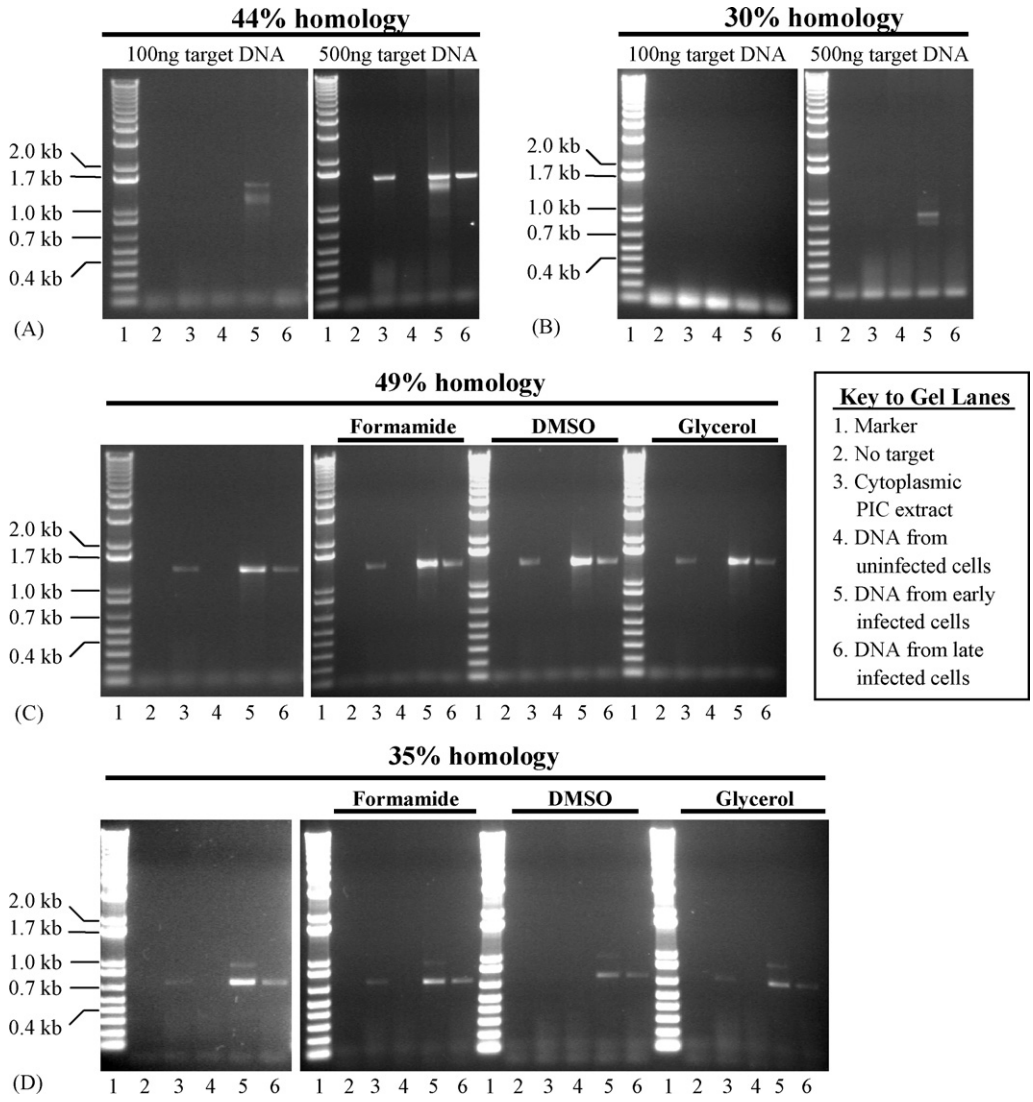

Fig. 3. PCR amplification of retroviral vector cDNA with variable target concentration or additives. Lanes are 1, marker; 2, no target; 3, cytoplasmic PIC extract; 4, DNA from uninfected cells; 5, DNA from early infected cells, 15 h post-addition of retroviral vector; 6, DNA from late infected cells, months following addition of retroviral vector. (A) SKSM2 retroviral vector transduced samples amplified with oKY356 and oKY214. The LTR is 44% of the total amplicon. Either 100 or 500 ng target DNA was added to each reaction. (B) SIN retroviral vector transduced samples amplified with oKY270 and oKY264. The LTR is 30% of the total amplicon. Either 100 or 500 ng target DNA was added to each reaction. (C) SKSM2 retroviral vector transduced samples (100 ng) amplified with primers oKY356 and oKY379 in the presence of no additive, 2.5% formamide, 5% DMSO, or 5% glycerol. The LTR is 49% of the total amplicon. (D) SIN retroviral vector transduced samples (100 ng) amplified with primers oKY325 and oKY264 in the presence of no additive, 2.5% formamide, 5% DMSO, or 5% glycerol. The LTR is 35% of the total amplicon length.

PCR reactions were repeated. With more target available, these primers were able to amplify the predicted 778 bp product from early infected DNA (Fig. 3B).

PCR fidelity has been shown to increase in the presence of formamide, DMSO, or glycerol (Bookstein et al., 1990; Landre et al., 1995; Sarkar et al., 1990). These additives all appear to increase amplification by reducing the formation of DNA secondary structures, but their ability to enhance PCR efficiency is variable depending on the template (Bookstein et al., 1990; Landre et al., 1995; Sarkar et al., 1990). The ability of formamide, DMSO, or glycerol to optimize 1LTR circle PCR was tested. DNA samples from the HIV-based vector SKSM2 with the full length LTR sequence were amplified with primers that yield an amplicon with 49% LTR sequence. In the absence of additives, these primers amplify specious products from PIC DNA and late infected DNA (Fig. 3C). The addition of 2.5% formamide, 5% DMSO, or 5% glycerol was not able to eliminate the amplification of these samples (Fig. 3C). Similarly, DNA samples from the SIN vector with a short LTR were amplified with primers that yield an amplicon with 35% LTR sequence. The additives had no effect on the fidelity of 1LTR circle PCR of SIN transduced samples (Fig. 3D). Other substances, such as betaine, detergents, and tetramethylammonium chloride, have been reported to enhance PCR amplification (Bachmann et al., 1990; Henke et al., 1997; Hung et al., 1990). The ability of alternative PCR additives to enhance the specificity of 1LTR circle detection by PCR has not been excluded.

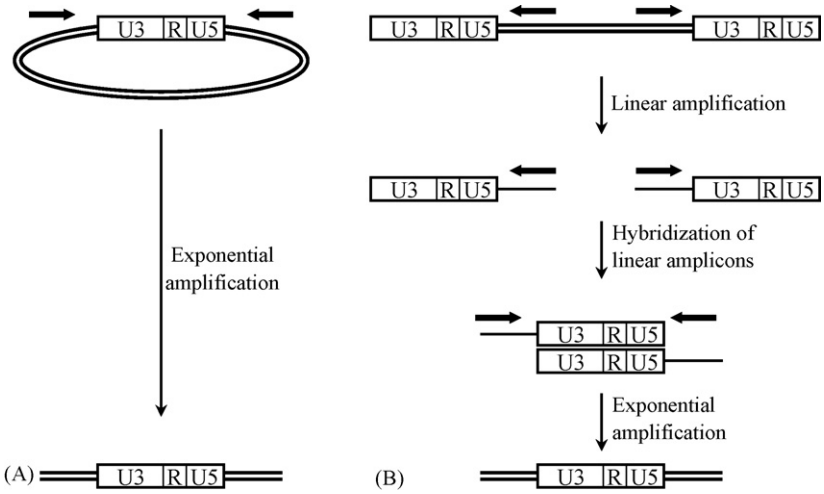

Fig. 4. Model of PCR amplification of retroviral vector cDNA. (A) Authentic exponential amplification of 1LTR circles. (B) Spurious amplification of a linear cDNA molecule proceeds first by linear amplification. The single stranded products of linear amplification are able to hybridize at the LTR sequences. These products are then exponentially amplified to yield a double-stranded product identical in size and sequence to the 'authentic' 1LTR PCR products. Double lines indicate double-stranded DNA, single lines indicate single stranded DNA.

It is likely that the primers used to amplify the 1LTR circle product are also able to amplify other cDNA forms, such as a linear cDNA molecule, first through a linear amplification (Fig. 4B). The linear single stranded DNA products accumulate and hybridize at the homologous LTR sequences. This hybrid molecule can serve as a target for exponential amplification yielding a product indistinguishable from the genuine exponentially amplified product based on either size or sequence.

PCR-based methods have become increasingly common for both quantitative and qualitative detection of viral sequences. For retroviruses such as HIV, it is possible to definitively amplify multiple cDNA forms, including 2LTR circles and integrated provirus which have unique amplicons. Amplification of 1LTR circles is complicated by the absence of a truly unique amplicon. Primers designed for 1LTR circle detection were able to amplify spurious products from other cDNA forms including linear cDNA molecules and integrated provirus. Such illegitimate amplifications were not reduced by the addition of formamide, DMSO, or glycerol, although other additives such as betaine or detergents have not been evaluated. Some primer sets appeared able to amplify authentic 1LTR circles without specious bands in control reactions. However, simply increasing the target concentration led to reappearance of unauthentic products in control reactions. This suggests that the amplification of true 1LTR circles is sensitive to subtle changes; this may include sensitivity to varying multiplicity of infection, which could further complicate the generation of appropriate control templates. Amplification of genuine 1LTR circles may be possible under precisely controlled conditions; however, since it is not possible to authenticate these PCR products by sequencing, detection of 1LTR circles by PCR remains challenging.

Acknowledgements

We thank members of the Fishel lab for helpful discussions. This work was supported by NIH grant CA56542 to R.F.

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