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Impact of the DNA extraction method on 2-LTR DNA circle recovery from HIV-1 infected cells

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Abstract

Detection of episomal 2-LTR DNA circles is used as a marker for the ongoing virus replication in patients infected with HIV-1, and efficient extraction of episomal DNA is critical for accurate estimation of the 2-LTR circles. The impact of different methods of DNA extraction on the recovery of 2-LTR circles was compared using mitochondrial DNA extracted as an internal control. The bacterial plasmid DNA isolation method extracted less than 10% of cellular DNA, 40% of mitochondrial DNA and 12-20 % of the input 2-LTR DNA. The total DNA isolation method recovered about 70% of mitochondrial DNA and 45% of the input 2-LTR DNA. The total nucleic acid isolation method recovered 90% of mitochondrial DNA and 60% of the input 2-LTR DNA. Similar results were obtained when the DNA was extracted from HIV-1 infected cells. Plasmid DNA isolation could not distinguish between 12 and 25 copies of 2-LTR DNA per million cells, whereas the total nucleic acid isolation showed a consistent and statistically significant difference between 12 and 25 copies. In conclusion, the total nucleic acid isolation method is more efficient than the plasmid DNA isolation method in recovering mitochondrial DNA and 2-LTR DNA circles from HIV-1 infected cells.

Keywords

2-LTR; HIV; Episome; Mitochondrial DNA; Retrovirus; PCR

1. Introduction

When HIV-1 infects a cell, its single-stranded RNA is converted into cDNA and then to double-stranded DNA that is integrated into the host chromosome. However, a fraction of the non-integrated HIV-1 DNA is converted into circular DNA, which contains two copies of the long-terminal repeat (LTR) and is referred to as 2-LTRs (Butler et al., 2002). The level of 2-LTR circles in cells has been shown to vary in patients depending on antiretroviral treatment, even though it is controversial whether the 2-LTR circles, once formed, are stable or labile (Morlese et al., 2003; Pierson et al., 2002; Sharkey et al., 2000). Accurate measurement of 2-LTR DNA is critical, as it may indicate the presence of newly infected cells (Buzon et al., 2010). The PCR method has been used most often as it is the only

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method that detects, both directly and sensitively, the 2-LTR circles in HIV-1 infected cells. However, this method requires a DNA extraction procedure that is suitable for PCR.

Since the 2-LTR circles exist as extrachromosomal DNA, the Hirt DNA isolation method suitable for extraction of low molecular weight DNA has been used to extract episomal DNA (Hirt, 1967; Miles et al., 2005; Ziegler et al., 2004). Plasmid DNA (pDNA) isolation methods, that remove bacterial genomic DNA by precipitation, leaving behind the smaller DNA in solution, have been the most commonly used methods for extracting 2-LTR DNA (Brussel et al., 2003; Buzon et al., 2011; Chavez et al., 2007; Gandhi et al., 2012; Gutierrez et al., 2011). Human mitochondria have circular episomal DNA of about 16.5 kbp. A few hundred copies of mitochondrial DNA (mtDNA) are present in every cell. It is hypothesized that any method that efficiently extracts mtDNA would also efficiently extract 2-LTR circles from virus-infected cells. The level of 2-LTR DNA is low in HIV-1-infected patients and is shown to be below detection in many patients undergoing antiviral treatments (Graf et al., 2011; Lam et al., 2012; Llibre et al., 2012). Therefore efficient extraction of episomal DNA is critical in obtaining accurate information on the level of 2-LTR DNA. In this study, a comparison of total nucleic acid (tNA), total DNA (tDNA) and bacterial plasmid DNA (pDNA) isolation methods on the extraction efficiency of episomal DNA by using mtDNA as a control, is reported.

Results show that the tNA method is superior to the pDNA isolation method for extracting 2-LTR DNA.

2. Materials and methods

2.1 Cells

Peripheral blood mononuclear cells (PBMCs) were obtained from HIV-1 positive study subjects after venipuncture or leukapheresis procedures, after signing informed protocol consents approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases. (Bethesda, MD). MT2 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10mM HEPES.

2.2 Preparation of 2-LTR DNA

Infection of MT2 cells with HIV-1 (NL4-3, 10 ng of p24/10⁶ cells) was carried out by spinoculation at 1200 × g for 2 h (Jiang et al., 2012). Cells were washed twice with PBS (pH 7.4), incubated at 37°C in a 5% CO₂ atmosphere and harvested at 48 h post-infection. After washing the cells twice with cold PBS (pH 7.4) aliquots of 3 million cells were used immediately for DNA extraction or stored at -80°C until they were used for DNA extraction. DNA was isolated by using Qiagen QIAamp DNA blood mini kit. The amount of 2-LTR DNA in this DNA preparation was determined by real-time qPCR using a plasmid containing 2-LTR DNA as the standard (see below).

2.3. Preparation of 2-LTR spiked PBMCs

PBMCs from EDTA-treated blood obtained from healthy donors were isolated by Ficoll-Hypaque (Lymphoprep™, Fresenius Kabi Norge AS, Norway) density gradient centrifugation according to the manufacturer's instructions and cell number was determined by counting with a hemocytometer. Twelve or 25 copies of 2-LTR DNA isolated from HIV-1 infected MT2 cells were added to aliquots of one million PBMCs and 500 or 2500 copies of 2-LTR circles were added to aliquots of 3 million PBMCs and stored at -80 °C until use.

2.4. DNA extraction methods

tNA was isolated using the Epicenter Masterpure DNA and RNA isolation kit (Madison, WI) from cells described above. The manufacturer's protocol, modified for samples of more than 2 million cells, was followed. Briefly, cells were lysed in lysis buffer and proteinase K treatment was extended to 45 min at 65°C to break down the cell membranes. Proteins were pelleted by a salting-out procedure and tNA was precipitated from supernatants by isopropanol and solubilized in 85 ul of 10 mM Tris buffer, pH 8.0. To obtain RNA-free nucleic acid, tNA was treated with 10 ug of RNase A and incubated at 37°C for 30 min as recommended by the manufacturer.

tDNA, including viral and mtDNA, was obtained by using Qiagen QIAamp DNA Blood mini kit (Valencia, CA) according to the manufacturer's protocol. Briefly, after lysing the cells with optimized buffer and enzymes, alcohol was added to the sample, and the lysate was loaded onto a QIAamp spin column and washed with wash buffer. The DNA was eluted with 85 ul of 10 mM Tris buffer, pH 8.0.

The third method employed to extract 2-LTR DNA was bacterial pDNA isolation, using two commercially available kits. DNA isolated by the Qiagen QIAprepSpin Miniprep Kit (Valencia, CA), is called QpDNA, and DNA obtained by the Roche High Pure Plasmid Isolation Kit (Indianapolis, IN) is known as RpDNA. The manufacturer's protocol for isolation of low copy number plasmids was used with the QIAprep Spin Miniprep Kit. Briefly, cells were lysed in alkaline lysis buffer and after clearing the solution of cell debris and chromosomal DNA; the episomal DNA was bound to silica-based gel membranes or glass fiber filter columns. Following the wash steps, the DNA was eluted with 100 ul of 10 mM Tris buffer, pH 8.0.

After extraction, the nucleic acid concentrations of the samples were quantitated using a NanoDrop 2000 Spectrophotometer.

The absolute level of mtDNA in the cells was obtained by using cell lysate as the source of DNA. Cell lysate was prepared by lysing the cells as described (Elbeik et al., 1997). Briefly, cells were suspended in 150 ul/million cells of buffer A (10 mM Tris-HCl, pH 8.0; 100 mM KCl; and 2.5 mM MgCl₂) and then lysed by adding 150 ul/million cells of lysis buffer B (10 mM Tris-HCl, pH 8.0; 2.5 mM MgCl₂; 1% Tween 20; and 1% NP40) containing 400 ug of proteinase K (Roche, Indianapolis, IN) per milliliter of buffer B. Cells were incubated at 65°C for 1.5 h, then proteinase K was inactivated by heating the lysate to 96°C for 15 min.

2.5. 2-LTR DNA circles quantitation by real-time PCR

tNA and tDNA samples isolated from 2-LTR spiked PBMCs, HIV-1 infected MT2, and PBMCs of HIV-1 positive donors were diluted to a concentration of 75 ng/ul in 10 mM Tris buffer, pH 8 and duplicates of 40 ul equal to 3 ug of nucleic acid were used for quantitative PCR. For the samples isolated by the QpDNA and RpDNA methods from 2-LTR spiked PBMCs, HIV-1 infected MT2, and PBMCs of HIV-1 positive donors, duplicates of 40 ul of eluent, equal to approximately 0.4-1.4 ug of DNA were added to the PCR reaction. The total 2-LTR circles copies in the tNA and tDNA samples was calculated by multiplying the copies in 3 ug by the total DNA yield and 2-LTR copies in QpDNA and RpDNA samples were estimated by multiplying the copies in 40 ul with the total elution volume (100 ul). A standard curve ranging from 6 to 124,000 copies per reaction was created for each run. A plasmid containing 2-LTR DNA (Yamashita and Emerman, 2004) was diluted in 10 mM Tris buffer, pH 8.0, containing 40 ng/ul of DNA isolated from healthy donor PBMCs and was run in triplicate as standard.

The following primers and probe were used: forward primer, MH535 (5'-AACTAGGGAACCCACTGCTTAAG-3'); reverse primer, MH536 (5'-TCCACAGATCAAGGATATCTTGTC-3'); and probe, MH603 [5'-(FAM)ACACTACTTGAAGCACTCAAGGCAAGCTTT-(TAMRA)-3']. (Butler et al., 2002). One hundred microliters of PCR contained 1×TaqMan universal master mix (Applied Biosystems, Carlsbad, CA), 600 nM forward and reverse primers, 150 nM probe, and 40 ul of DNA. After initial incubations at 50 °C for 2 min and 95°C for 10 min, 45 cycles of amplification were carried out for 15 s at 95°C followed by 1 min at 60 °C. All PCRs were carried out in an optical 96-well plate used for Applied Biosystems' 7900 Real-Time PCR system.

2.6. Quantitation of mtDNA by real-time PCR

Quantitation of mtDNA in tNA, tDNA, QpDNA, and RpDNA samples was carried out by real-time PCR using the mitochondrial cytochrome b (mtCytB) gene as described earlier (Chiappini et al., 2004). Duplicates of 375 pg of tNA and tDNA and duplicates of 5 ul of 1:1000 diluted (10 mM Tris buffer, pH8) QpDNA and RpDNA were used for mtCytB PCR. A standard curve ranging from 50 to 50×10^6 copies in triplicate was created using 10-fold serial dilutions of a purified PCR product generated using the following primers: forward, 5'-AACTACAAGAACACCAATGACCCC-3', reverse, 5'-GCCGATGTTTCAGGTTTCTGAG-3'.

2.7. Statistical analysis and calculations

The results from at least three replicates were expressed as mean $\pm$ SD. The p-value, calculated using Student's t-test, was considered statistically significant if it was less than 0.05.

3. Results

3.1 Comparison of tNA, tDNA, and pDNA isolation methods for the recovery of episomal DNA and 2-LTR in 2-LTR spiked PBMCs

For each method, DNA was isolated from replicates of six PBMC samples containing either 500 or 2500 copies of 2-LTR circle DNA per three million cells. Results presented in Fig.1A show the total DNA yield isolated from three million cells. An average of 19 and 16 ug of nucleic acid was isolated by tNA and tDNA methods, respectively, while pDNA isolation, as expected, yielded the least amount of DNA, an average of 1.4 ug per 3 million PBMCs. DNA yield for pDNA was lower than the tNA and tDNA because it excludes most of the high molecular weight nucleic acids. If the pDNA isolation method had extracted all episomal DNA, one would expect approximately equal amounts of mtDNA in each of these preparations. The recovery of mtDNA was assessed by comparing the copy numbers of mtCytB, which was quantified by simultaneously processing all the isolates by real-time qPCR. tNA and tDNA methods recovered about 90% and 70% of mtDNA, respectively, whereas pDNA isolation recovered about 40% (Fig.1B). The absolute level of mtDNA in the samples was obtained with the DNA prepared by lysing the cells without any precipitation steps (Elbeik et. al., 1997). Fig.1B (LB A/B) shows the absolute number of the mtDNA in the lysate. mtDNA copies per PBMCs ranged from 700 to 800, and that is in line with published estimates (de Mendoza et al., 2004).

Next, the recovery of episomal 2-LTR circles extracted by different methods was estimated by real-time PCR. Results showed that tNA and tDNA have about 62% and 45%, respectively, of input 2-LTR DNA. However, in QpDNA samples, only 18% of input 2-LTR DNA was recovered (Fig. 1C). The yields of both 2-LTR DNA and mtDNA were lower in pDNA preparations than in the other two methods. This result is supported by the data in

Fig. 1D showing that the levels of 2-LTR DNA per mtDNA copy were similar for all methods tested.

3.2 tNA and tDNA isolation methods recover more of the episomal DNA than the pDNA isolation method from HIV-1 infected MT2 cells

Next, different DNA isolation methods were evaluated for efficiency of 2-LTR DNA extraction by isolating episomal DNA from HIV-1 infected cells. Three million HIV-1 infected MT2 cells were processed for DNA isolation. To confirm the poor recovery results using the pDNA isolation method, we included an additional bacterial plasmid isolation kit from a different manufacturer. Triplicates of the samples were used for each method. A comparison of nucleic acid recoveries in these cells (Fig. 2A) showed results that were similar to those using 2-LTR DNA added to PBMCs. The levels of mtDNA in both pDNA preparations were an average of 3 times lower than in tNA and about 2 times lower than in tDNA preparations (Fig. 2B). Similarly, both pDNA isolates had the lowest levels of 2-LTR circles, recovering only about 10%-15% of the 2-LTR DNA compared to tNA (Fig. 2C), and the pDNA isolation method also recovered a lower level of mtDNA than the tDNA method. Normalization of the 2-LTR DNA level by mtDNA confirms this conclusion (Fig. 2D). Overall, the data show that the tNA method consistently yielded high amounts of total, as well as mitochondrial and 2-LTR, DNA.

3.3 Comparison of low copies of 2-LTR recovery by the tNA and pDNA methods

Patients undergoing antiviral therapy have been shown to have low-to-undetectable amounts of 2-LTR in their cells (Graf et al., 2011; Lam et al., 2012; Llibre et al., 2012). Next, the efficiency of the tNA and pDNA methods in recovering 2-LTR circles from cells containing 12 or 25 copies of 2-LTR DNA per million cells was compared. Fig. 3 shows the summary of the results obtained by the two different methods using replicates of 24 samples. The tNA method recovered most of the input 2-LTR DNA. In contrast, the pDNA isolation recovered an average of 1.5 and 3 copies from samples containing 12 and 25 copies, respectively, which was not indistinguishable statistically. These results demonstrate that the tNA method is better than the other methods tested for isolating 2-LTR and mitochondrial DNAs.

DNA was isolated from PBMCs of three HIV positive individuals using the tNA and pDNA methods and Table 1 shows the level of 2-LTR circles and mtDNA in these preparations. As expected, the pDNA method extracted only 8 to 12% of the mtDNA compared to the tNA. Two out of three pDNA preparations were negative for 2-LTR DNA whereas all three tNA preparations had 2-LTR DNA, confirming that the tNA method is superior to the pDNA method for extracting 2-LTR DNA.

4. Discussion

2-LTR DNA has been used as a marker for HIV-1 persistence in patients. A number of studies have shown that the 2-LTR DNA level is indicative of ongoing virus replication, and the presence of 2-LTR circles could be an indication of ongoing de novo infection (Butler et al., 2002; Buzon et al., 2011; Dornadula et al., 2001; Panther et al., 1999; Pauza et al., 1994; Sharkey et al., 2005). Therefore, an accurate estimation of 2-LTR circles in patient samples is essential to monitor viral replication and develop or change the treatment strategy. It is well established that once the patient undergoes a long and an effective antiviral treatment, the level of 2-LTR circles could become very low or undetectable (Graf et al., 2011; Lam et al., 2012; Llibre et al., 2012). However, the impact of DNA isolation techniques on the 2-LTR DNA recovery has not been explored. The selection of the most effective method for obtaining a sufficient amount of nucleic acids is critical, and to date no information is available on the best way to process samples to obtain optimal 2-LTR DNA recovery. The

bacterial plasmid DNA isolation method has been the most commonly used method for isolating 2-LTR circles from patient samples (Cara et al., 2004; Cheng et al., 2008; Friedrich et al., 2010; Luzuriaga et al., 2000). Eliminating high molecular weight nucleic acids from the sample, which might inhibit PCR, could enrich the episomal DNA and also improve PCR sensitivity. However, results presented in this study clearly show that the bacterial plasmid isolation method did not extract all episomal DNA and had the lowest level of 2-LTR DNA recovery. In addition, the tDNA isolation method, the second most commonly used method (Graf et al., 2011; Koelsch et al., 2011; Saenz et al., 2004) for 2-LTR DNA purification, which is based on using a silica column, had poorer recovery of small molecular weight DNAs than the most efficient tNA isolation method, which uses an organic extraction and precipitation approach.

It is known that mtDNA is coated extensively with proteins and packed into nucleoids (Bogenhagen, 2012) and 2-LTR DNA is present as a nucleoprotein complex in the nucleus (Sloan and Wainberg, 2011). tNA and tDNA extraction methods incorporate proteases to digest the proteins, which may facilitate better recovery of episomal DNA. In the plasmid DNA isolation method, cells are lysed with an alkaline buffer that may not break down cellular membranes, and the addition of chaotropic salts and acetate-containing neutralization buffer to the incompletely denatured nucleoprotein complex may precipitate 2-LTR circles along with the genomic DNA to be discarded, leading to the loss of much of the 2-LTR DNA.

In order to increase the detection sensitivity of episomal DNA from patients with low levels of 2-LTR circles, greater amounts of nucleic acids have to be added to the PCR. However, it is known that an excess of DNA may inhibit the PCR process (Okello et al., 2010). But, addition of up to 3 µg of nucleic acid did not have a significant influence on PCR efficiency. With this approach, one could estimate the level of 2-LTR in 500,000 cells in a single reaction.

The data presented in this study show that mtDNA could be used as an internal control to calculate the efficiency of episomal DNA recovery. By estimating the total mtDNA level using a cell lysate and comparing the level of mtDNA in DNA samples, the efficiency of the episomal DNA extraction may be calculated. The mtDNA copy number varies between the cell types, and also is correlated with viral load, disease stage and antiviral treatments (Chiappini et al., 2004; Kajander et al., 2002; Miura et al., 2003). However, it would be useful to test for mtDNA within the same specimen to ensure confirm adequate 2-LTR DNA recovery.

5. Conclusion

Among the tested methods, the tNA isolation method recovered most of the 2-LTR DNA as well as most of the mtDNA. The level of 2-LTR circles in HIV-1 infected cells could be estimated accurately by using mtDNA levels in the samples as an internal control.

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Highlights

- Plasmid DNA isolation method used by many laboratories is inefficient in extracting 2-LTR DNA
- Most of the episomal DNA including the 2-LTR circle DNA is extracted by total DNA isolation method
- DNA isolated by plasmid DNA isolation method could not distinguish between 12 and 25 copies of 2-LTR
- Total nucleic acid isolation method could detect a significant difference between 12 and 25 copies of 2-LTR
- Mitochondrial DNA could be used as a recovery control for 2-LTR circles in the DNA isolation process

Fig.1. The pDNA isolation method yielded the least amount of episomal DNA and 2-LTR in 2-LTR spiked PBMCs

DNA was isolated from six replicates of three million PBMCs supplemented with either 500 (Columns 1, 3, and 5) or 2500 (Columns 2, 4, and 6) copies of 2-LTR circles. (A) Total DNA yield (ug). (B) mtDNA copies extracted from 3 million cells. DNA from control (Column 7) sample was prepared by the cell lysis method as described in Materials and Methods. (C) 2-LTR circles extracted from 3 million cells. (D) 2-LTR circles per 10⁸ mtDNA.

Fig.2. Comparison of the 2-LTR and mtDNA levels in nucleic acids isolated by different methods from MT2 cells infected with HIV-1

(A) Total DNA yield (ug) from 3 million cells. (B) mtDNA copies extracted from 3 million cells. (C) 2-LTR circle levels in DNA isolated by different methods from 3 million MT2 cells infected with HIV-1. (D) 2-LTR circles per 10⁸ mtDNA. Data represent the replicates of three isolations.

Fig.3. The pDNA isolation method could not distinguish between 12 and 25 copies of 2-LTR per million cells

DNA was isolated from one million PBMC samples supplemented with 12 (Columns 1 and 3) and 25 (Columns 2 and 4) copies of 2-LTR DNA. The data represent the replicates of 24 isolations.

Table 1

Quantification of 2-LTR DNA in patient's PBMC

Patient number	2-LTR copies/ 10^6 cells		mtDNA/cell	
	tNA	QpDNA	tNA	QpDNA
1	308	2 ^a	600	49
2	43	ND	242	21
3	5 ^a	ND	1311	166

tNA and QpDNA were isolated from one million PMBCs from HIV positive individuals and 2-LTR and mtDNA were quantitated.

^a2-LTR copy numbers were lower than the lowest standard ND, Not Detectable