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# Low stringency-PCR (LS-PCR) allows entirely internally standardized DNA quantitation

**Otavia L. Caballero, Luisa L. Villa* and Andrew J. G. Simpson[1]**

Ludwig Institute for Cancer Research, Rua Prof Antonio Prudente 109 — 4th floor, 01509-010 São Paulo, Brazil, and [1]Centro de Pesquisas René Rachou, Fiocruz, Belo Horizonte, MG, Brazil

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The quantity of DNA in a sample can be estimated using the polymerase chain reaction (PCR) by comparing the amounts of products that result from the co-amplification of a target sequence and an added internal standard of known concentration that is amplified by the same primers (1–5). In several important applications of competitive PCR, such as the detection of gene amplification in tumors and the quantification of some viral infections, it is the ratio of the number of copies of the target gene to the number of diploid genomes present that is in essence being sought. In achieving this value by competitive PCR there are several points at which error can occur including inter-tube variations in the amount of added standard and exact quantification of the amount of DNA in the sample and variable degrees of sample degradation. We here show that these variables, as well as the need for construction of an internal standard, can be eliminated by using Low Stringency-PCR (LS-PCR) for DNA quantification. In LS-PCR, a specific primer pair is employed under low stringency conditions so that in addition to the specific product a series of other DNA fragments are amplified that result from the low stringency interaction of the primers with other regions of the DNA in the reaction tube (6–8). In the case of the application of LS-PCR to the diagnosis of infection by microorganisms, the low stringency products (LSPs) are invariably amplified from the host DNA due to the relative molecular masses of the genomes of the two organisms. Inspection of our previous data shows that there is competition between the specific product and the LSPs although, in the latter case, the actual sequences being amplified are entirely unrelated to the specific product and indeed may have the same primer at both extremities. We have now found that this competition allows quantification of the specific product in relation to the amount of host DNA present without the need for constructing an internal standard or exact quantitation of the overall amount of DNA in the sample.

The experimental system we have used is that of Human Papillomavirus which is responsible for anogenital cancer (9). It has been suggested that the number of viral genomes present in the infected cells may correlate with the severity and or progression of disease (10,11), thus prompting our interest in attempting to develop a simple but precise method of determining viral copy number.

A reference plasmid containing the entire HPV-16 genome (kindly provided by Dr E.M. de Villiers, Deutsches Krebsforschungszentrum, Heidelberg) was mixed with normal human DNA and subjected to LS-PCR using a primer pair specific for the L1 gene of several HPV types (12). A complex profile was produced consisting of the specific band of ~140 base pairs (bp) and many LSPs ranging from <100 bp to more than 1Kb (Fig. 1A).

We measured the relative amounts of the specific HPV product and one of the LSPs (HUM1) generated by LS-PCR amplification from three different quantities of plasmid–human DNA mixtures at four plasmid copy numbers (Fig. 1B and C). Inspection of the gel and the log/log plot of the relative intensities of the two bands against copy number reveals that the internal competition for amplification is directly related to the relative amount of the specific target and that the outcome of the competition is constant over a four fold variation in the amount of DNA in the reaction. Further experiments showed that inclusion of up to 200 ng of human DNA (i.e. 40-fold variation in DNA concentration) did not alter the relationship between the amplification of specific HPV product and HUM1 over the range of copy numbers shown (data not shown).

The experiment shown in Figures 1B and C was repeated with three other primer pairs that amplify HPV 16 DNA. In all cases, competition between amplification of the specific band and the LSPs was observed. Obviously the pattern of LSPs varies as a function of the primer pair and GP5/GP6 was selected for further investigation due to the clear pattern of well amplified LSPs that they produce.

Figures 1D and E illustrate the use of LS-PCR to estimate HPV copy number in two cervical carcinoma cell lines containing integrated HPV genomes at different copy numbers (HeLa, 20–40 copies of HPV-18; Caski, 400–600 copies of HPV-16) (13) where different amounts of the cell line DNA were compared with duplicate samples of plasmid-human DNA standards. Our estimates of the copy numbers, (49 for HeLa and 416 for Caski) which are in excellent agreement with results and again show read out independence from the amount of DNA present in the sample. In addition, the experiment illustrates the fact that the HUM1 product is uniformly amplified from any human DNA (verified with more than 25 randomly selected human DNA preparations) and that the method functions with different HPV types (data not shown).

The data shown demonstrate that a usable quantitative read out is achieved from LS-PCR despite the fact that the LSP used for quantification is presumably completely unrelated to the specific target and that the amplification is driven to the plateau. To test the importance of LSP choice, a second LSP (HUM2 in Fig. 1A)

* To whom correspondence should be addressed

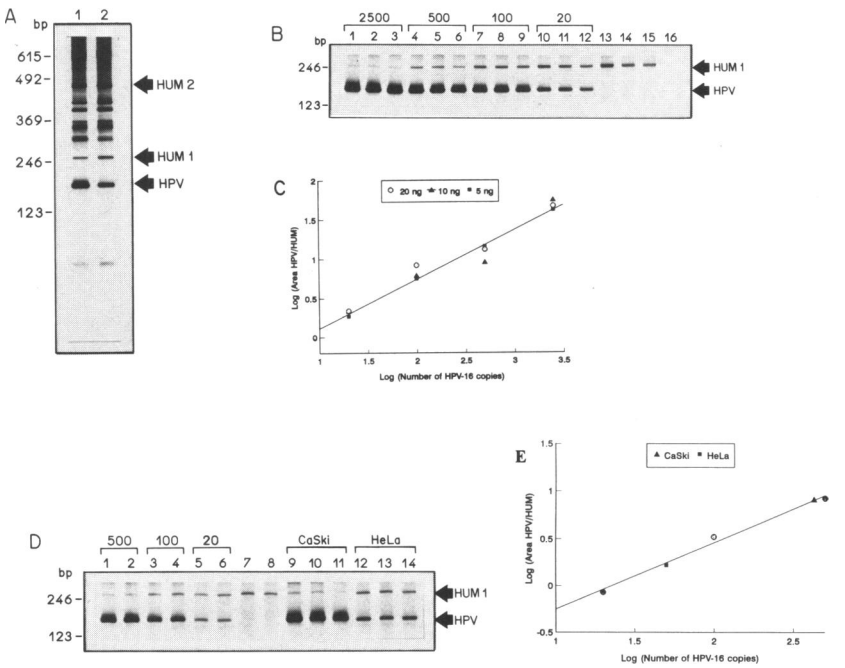


**Figure 1.** (**A**) Two (lane 1) and 0.4 pg (lane 2) of a plasmid harboring the entire HPV-16 genome were mixed with 10 ng of human DNA extracted from normal mammary gland tissue. One µl of this mixture, corresponding to 100 and 20 copies of HPV-16/cell (11), was amplified in a final volume of 10 µl with 10 pmol of each primer (GP5 = TTGTTACTGTGGTAGATTAC and GP6 = GAAAAATAAACTGTAAATCA) (12), 200 µM of each deoxyribonucleotide, 0.8 U of *Taq* DNA polymerase (kindly provided by CENBIOT, Brasil), 50 mM KCl, 3.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3). The mixture was overlaid with a drop of mineral oil to prevent evaporation and submitted to the following cycle program: one cycle at 94°C for 3 min; 10 cycles of 92°C for 30 s, 45°C for 1 min and 72°C for 1 min; 30 cycles at 92°C for 30 s, 40°C for 1 min and 72°C for 1 min; 1 cycle at 72°C for 5 min. Eight µl of the products were analysed by electrophoresis on a 8% polyacrylamide gel followed by silver staining (14). The specific amplification products (HPV) and LSPs used for quantification (HUM1 and 2) are arrowed. (**B**) Various amounts of the HPV-16 plasmid were added to 20 ng (lanes 1, 4, 7 and 10), 10 ng (lanes 2, 5, 8 and 11) and 5 ng (lanes 3, 6, 9 and 12) of human DNA to produce mixtures corresponding to 20, 100, 500 and 2500 copies/cell, and amplified as described above. Lanes 13–15: 5, 10, 20 ng of human DNA with no HPV DNA added; lane 16, no DNA added. (**C**) Plot of the quantitative analysis of the electrophoretic pattern shown in B. The logarithm of the ratio between the area of the HPV (140 bp) and HUM1 (~260 bp) bands (determined by laser densitometry, UltroScan XL, Pharmacia/LKB) was plotted against the logarithm of the HPV copy number. The results obtained with each amount of human DNA are indicated. (**D**) LS-PCR quantification of HPV genomes present in cervical carcinoma cell lines. Different amounts (20, 10 and 5 ng) of total DNA extracted from Caski (lanes 9–11), and HeLa (lanes 12–14) cell lines were submitted to LS-PCR as described. A calibration curve was run in the same experiment, with plasmid–human DNA mixtures corresponding to 500 (lanes 1 and 2), 100 (3 and 4), 20 (5 and 6), and no (lanes 7 and 8) copies of HPV-16 (human DNA, 10 ng). (**E**) Plot of the data obtained in D. The points for the standard curve are the average of duplicates, and for cell lines the average of three samples containing 20, 10 and 5 ng.

was selected arbitrarily for the construction of a standard curve (data submitted, not shown). Again a linear relationship was found indicating that the outcome of competition under these conditions is not apparently dependent of the nature of competitive fragment. Further testing of two other LSPs between HUM1 and 2 also resulted in a linear relationship although in each case the slope of the curve differed slightly. There was no relationship between slope (and hence accuracy) and LSP size or intensity. In general, we conclude that LSP selection should be made empirically although from a practical viewpoint proximity of LSP to the specific band is advantageous. We also investigated the importance of PCR cycle number and found that HPV/HUM1 ratio remained constant when measured between 28 and 40 cycles (data submitted, not shown).

We propose that LS-PCR is the simplest and most perfectly controlled method of undertaking quantitative competitive PCR in situations where target sequence copy number per cell is required. The methodology avoids the necessity of accurate quantification of the amount of DNA in the sample and of constructing a competitive DNA fragment to be added to amplification tubes as an internal standard.

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