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INCREASED SENSITIVITY OF INTEGRATED HIV IN GENOMIC DNA: TOWARDS A NON EXPONENTIAL AMPLIFICATION BASED METHODOLOGY.

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1. INTRODUCTION.

The human immunodeficiency virus (HIV) has been demonstrated to be the etiological agent of acquired immune deficiency syndrome, AIDS (1). In the host cell, retroviral DNAs exist in three main forms: unintegrated linear, unintegrated circular, and integrated (the provirus). High levels of unintegrated forms of retroviral DNA often correlate with superinfection and accompany the cytopathic effects. In culture, HIV-1 infection also results in high levels of unintegrated viral DNA although direct correlations with cytopathicity have not been proven. Each of the three species of viral DNA, can be detected in blood and brain of AIDS patients and, in the case of autopsy samples from patients with HIV encephalitis, there is a considerably higher proportion of unintegrated viral DNA (2).

Several isolates of HIV have demonstrated to be divergent in sequence; however some regions such as *gag* show a high degree of homology, and can thus be used in molecular diagnostic (1).

The PCR (Polymerase Chain Reaction) technique, is the most sensitive method to determine viral sequence, since it can increase the target by 10^6 times. However, in spite of the detection of viral DNA at levels far below that possible by other hybridization methods PCR is, in its conventional form, of limited use for quantitative analysis (3).

1.1 Detection of HIV.

The initial screening procedure entailed the detection of circulating antibodies using ELISA (enzyme-linked immunosorbant assay). Samples that test positive are subsequently screened for reactivity to specific viral antigens *via* a Western blot assay. These tests can only detect HIV-1 in patients who have circulating antibodies and/or viral antigens, and are not 100% reliable (5). In fact, the appearance of detectable serum antibodies may occur weeks or even months after infection, thereby diminishing the utility of serologic tests for early detection of infection. Additionally, infectious virus can only be isolated from about 50% of individual in certain seropositive groups. Other assays commonly used as secondary HIV-detection systems include (i) Mg^{+}-dependent reverse transcriptase activity, (ii) transmission of virus to non-infected tissue culture cells, and (iii) detection of the virally encoded *gag* protein. Each of these assays has some limitations due to their lack of sensitivity (1).

Direct detection of HIV-1 nucleic acid sequences in peripheral blood samples, from either AIDS or ARC (AIDS-related complex) patients, using Southern blot analysis or *in situ* hybridization, is extremely inefficient. In fact, less than 0.01% (1/10,000) of the lymphocytes in mononuclear cell preparations from AIDS and ARC patients has been shown to harbor HIV-1 nucleic acid sequences (1).

Given this very low number of peripheral blood lymphocytes expressing viral RNA, PCR DNA amplification of HIV-integrated DNA has been introduced to increase the sensitivity (6).

Murakawa and et al (1), developed a methodology that enables the rapid direct detection of HIV-1 sequences from 10 to 15 ml samples

of peripheral blood without an intermediate culturing step. This assay was based on PCR amplification, followed by Southern blotting analysis. They included a novel contribution to the PCR methodology involving a transcriptional step, in which the targeted region is amplified over 50-fold using T7 RNA polymerase.

Other approaches to detect viral DNA, are based on the detection by hybridization with ^{32}P-labeled DNA probe of a portion of the amplified fragment, which is made specific with a subsequent step. Specific restriction endonuclease cleaves the resultant hybrid, which is analyzed in a polyacrylamide gel to locate the specific HIV-1 diagnostic fragment. Ou and et al (4), used this protocol to amplify specific regions of HIV-1 provirus present, either as the free episomal form, or in the integrated form in patient's chromosomal DNA. By means of PCR, proviral sequences of the human immunodeficiency virus (HIV-1) were identified directly in DNA isolated from peripheral blood mononuclear cells (PBMCs) of persons seropositive, but not in DNA isolated from PBMCs of persons seronegative for the virus. Primer pairs from multiple regions of the HIV-1 genome were used, to achieve maximum sensitivity of provirus detection. HIV-1 sequences were detected in 100% of DNA specimens from seropositive patients from whom the virus was isolated by coculture, but in none of the DNA specimens from a control group of seronegative, virus culture-negative persons. However, in comparison with cocultivation of peripheral blood mononuclear cells methods, HIV-1 sequences were detected in 64% of DNA specimens from seropositive, virus culture-negative patients. This method of DNA amplification made it possible to obtain results within 3 days, whereas virus isolation takes up to 3 to 4 weeks. The method may therefore be used to complement, or replace, virus isolation as a

routine means of determining HIV-1 infection. They supposed three possible explanations for the identification of persons, who were seropositive, but both virus culture-negative and PCR-negative. First, these persons may have contained an insufficient number of provirus copies (that is, very few infected lymphocytes) to be directly detected by the PCR technique described by them. Second, these persons may have been infected with HIV-1 containing genetic variations or deletions in the regions targeted for amplification. Third, these persons may not have harbored HIV-1 proviruses.

It has also been demonstrated that DNA extracted from formalin-fixed paraffin-embedded tissue, is suitable as a template for the detection of HIV provirus using PCR. This method offered some advantages: 1) formalin fixation and paraffin embedding render the tissue non-infectious, and 2) routinely obtained biopsy and postmortem material can be studied, (7). However, the low frequency HIV-1-infected cells in patients, has made it difficult to determine the structure of the viral DNA in fresh tissue samples, from AIDS patients by standard methods such as Southern hybridization.

Byrne and et al (8), extended the application of PCR into a specific assay for RNA sequences, a measure of active infection for detection of retroviral genomes or virus-specific mRNA. They detected proviral DNA copies in cell cultures of HIV+ patient samples. Reverse transcription/PCR detection of HIV RNA was 10^4 times more sensitive than detection of unamplified material using a slot blot and a ^{32}P primer-extended probe.

Also, Arrigo and et al (9) could detect low-abundance HIV-1 RNAs transcripts in lymphoid cells, employing the PCR method with RNA isolated from HIV-1-infected cells by first converting the RNA to cDNA with reverse transcriptase. They used an end-labeled

oligonucleotide primer and decreased the number of cycles of PCR from 40 to 25, also eliminating the transfer and hybridization steps of the conventional PCR method, which is both extremely sensitive and quantitative. They estimated that the sensitivity of this method is more than 10^4-fold that of standard RNA detection systems. It was possible detected low-abundance HIV-1 RNAs from 10^7 cells.

Using a double polymerase chain reaction, which allows the detection of single molecules of provirus, and a method of quantification of the provirus molecules, it has been possible to measure provirus frequencies in infected individuals down to a level of one molecule per 10^6 mononuclear cell. Only a small proportion of mononuclear cells contain provirus (median value of samples from 12 patients, one per 8,000 cells) and most if not all of the infected cells, carry a single provirus molecule. The frequency of provirus-carrying cells correlated positively both with the progression of the disease and with the success with which virus could be isolated from the same patients by cocultivation methods. The method proposed by these authors was based on a modified PCR method with a sensitivity sufficient to detect a single molecule of target DNA. After a first PCR amplification with a pair of HIV-specific primers, a small proportion of the product was used as the template for a second round of PCR amplification using a second set of primers nested within the first pair. They suggest that combining this double PCR method with a limiting dilution approach, both the proportion of infected cells and the number of molecules of HIV provirus per cell can be accurately estimated. Results obtained with PBMCs from 12 HIV-positive hemophiliacs showed a wide range in the proportion of provirus-carrying cells, with a median value of one provirus in about 8,000 cells (10).

Ampli-Vax™ matrix, which maintains separate template and primers from Taq DNA polymerase until the start of the first PCR cycle, has recently been demonstrated to increase the specificity of PCR. However, in the tested conditions, 40 cycles of amplification are still required, due to the very small number of starting HIV copies: in fact, when PCR starting substrate is 100 ng of genomic DNA with 1 copy of HIV/5.000 cells, about 3 copies of viral DNA are amplified until the production of hundreds of nanograms or micrograms of amplified fragment, increasing consequently the risks of PCR carryover (32).

1.2 Disadvantages of the PCR amplification.

The PCR amplification has been widely used for HIV diagnosis, because this technique is highly sensitive, but it presents serious disadvantages: 1) Presence of false positive: using many number of cycles, the risk of carryover is higher. 2) There is low specificity of reaction with very rare substrates, such as integrated HIV-1 and 2 that are present in 1/5.000-1/10.000 lymphocytes (10).

Furthermore, the concentration of a specific sequence in a sample can influence the relative homogeneity of the PCR products. Thus, a single copy nuclear gene present twice in every diploid cell, can usually be detected as a unique band after gel electrophoresis of the PCR products; but amplifications of a sequence present in only one of 10,000 cells is likely to yield a more heterogeneous gel profile (11). In other words, when the target to be amplified is very rare, the specificity of PCR is very low, therefore Southern blotting is required after a high number of PCR cycles, to detect the amplification

products. This has been usually the case for HIV, where it is possible to find 1 copy of HIV in 5.000 or more cells.

Thus a method is: 1) sufficiently sensitive, 2) fast, 3) not based on any "exponential" amplification step; should be considered very useful.

Contamination of PCR Reactions (Carryover).

Because the PCR can generate trillions of DNA copies from a template sequence, contamination of the amplification reaction with products of a previous PCR reactions (product carryover), exogenous DNA, or other cellular material, can create problems both in research and diagnostic applications. In general, attention to careful laboratory procedures _ pre-aliquoting reagents, the use of dedicated pipettes, positive-displacement pipettes, or tips with barriers preventing contamination of the pipette barrel, and the physical separation of the reaction preparation from the area of reaction product analysis _ minimizes the risk of contamination (31). Several approaches to minimize the potential for PCR product carryover have been developed, all based on interfering with the ability of the amplification products to serve as templates. For instance, by using sodium hypochlorite (Clorox) (12), or using the exonuclease III (13). Also, photochemical procedures has been proposed (14), treatment with DNase I before adding template (15), the use of psoralen/UV treatment (16), or the use of γ irradiation (17).

A recently proposed alternative has been by using the product of the *Escherichia coli ung* gene (UDG), which removes the uracil residues from the sugar moiety of either single-or-double-stranded DNA. This method involves substituting dUTP for dTTP in all PCRs to ensure that all DNA arising from these amplifications will contain dU

(dU-PCR). If UDG is included as a component of subsequent PCRs and the reaction is incubated or 10 min at 37°C, then any contaminating dU-DNA in the reaction will be digested. After, the UDG can then be inactivated by treatment at 95°C. Although UDG is an effective reagent to control carryover contamination in PCRs, residual UDG activity can potencially degrade dU-PCR products under certain conditions. (19).

Isaacs and et al, have developed an post-PCR sterilization process, which is based on isopsoralen photochemistry method, have been applied to the procedure of diagnostic assay for HIV-1 (18). However, this added step, made more difficult the detection during the diagnosis.

1.3 Paramagnetic Particles.

The recent introduction of high_quality paramagnetic particles offers new possibilities for molecular separations. Attaching specific functional groups to these beads, such as a protein or oligonucleotide, allows binding of target molecules in solution that can then be manipulated according to both their magnetic and chemical properties (20).

Fry and et al (21), have described the use of paramagnetic particles for DNA purification, specially for sequencing applications. Previous limitations in purifying molecules of interest may be overcome by strategies employing paramagnetic particles because, they are readily adaptable to automation. Paramagnetic particles eliminate the need for centrifugation, filtration and precipitation. The

particles also greatly reduce the volume size of the whole hybridization process because of their high reaction surface area.

The DNA isolated using this technology, is suitable for all molecular biology applications. This technology make use of a biotinylated primer to hybridize at high efficiency in solution to the respective sequence. The hybrids are captured and washed at high stringency, using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The hybridized DNA is recovered from the solid phase by the simple addition of water and heating at 80°C for few minutes.

1.4 The Ligation Detection Reaction (LDR), and Ligation chain Reaction (LCR).

The cloning of a thermostable ligase has opened the possibility to amplify DNA, through of LDR and LCR protocols (22, 23, 24 and 26), which are based on the ability of two oligonucleotides to anneal to regions immediately adjacent to each other on a complementary target DNA molecule, and then joined covalently by the action of a DNA ligase (23). The LCR technique employs four oligonucleotides, two adjacent oligonucleotides which uniquely hybridize to one strand of target DNA and a complementary set of adjacent oligonucleotides, which hybridize to the opposite strand.

The LDR only uses two oligonucleotides, using the same principle as LCR, but the target is linearly amplified. Thermostable DNA ligase, which joins the two juxtaposed oligonucleotides by the formation of a phosphodiester bond (25, 23), will covalently link each set provided that there is complete complementarity at the junction. Since the oligonucleotide products from one round may serve as substrates

during the next round, the signal is amplified exponentially, in the case of LCR, which is analogous to PCR amplification, but linearly in the case of LDR. A single-base mismatch at the oligonucleotide junction will not be amplified and can therefore be distinguished. A second set of mutant-specific oligonucleotides are used, in a separate reaction to detect the mutant allele (12).

In contrast to gene detection techniques based on immobilizing the target DNA, such as DNA blots, the hybridization reported using this method, is preformed in solution and in a small volume, which reduces the time required for hybridization. It also avoids the step of immobilizing the target DNA. Both ligation and binding of the biotinylated oligonucleotides are efficient and rapid steps that allow for quantitative detection of target molecules (23).

Specificity of LCR and LDR.

The specificity of ligation can be particularly enhanced by performing the reaction at or near the melting temperature (Tm) of the two oligonucleotides. PCR amplifications exploits two primers to obtain three types of information: 1) presence of target sequence, 2) distance between primers, and 3) sequence present between primers.

LCR exploits four primers while LDR two primers only; they obtain two types of information: 1) presence of adjacent target sequences, and 2) presence of perfect complementary to the primers at the junction of these sequences. PCR, LCR and LDR amplification, derive their specificity from the initial target DNA. This specificity is enhanced by: 1) use of oligonucleotides of sufficient length to uniquely identify individual humans or the target genome, and 2) use of the temperatures near to the Tm of oligonucleotide .

With PCR, background target-independent amplification results also in primer dimers, which are of lower molecular weight and thus easily distinguished. However, with LCR and LDR, background target-independent amplification yields the same size product. In order to make LCR and LDR available in the laboratory practice (as for PCR), it was necessary to eliminate target independent ligations completely. This was accomplished with the use of a thermostable ligase (12).

Thermostable DNA Ligase.

Thermostable DNA Ligase, which is derived from a thermophilic bacterium, catalyzes NAD-dependent ligation at temperatures from 15°C up to the denaturation temperature upon the nature and temperature stability of the DNA structure to be ligated. The half-life of the enzyme is greater than one hour at 95°C. If the nucleotides at the ligation junction are not correctly base-paired with the template DNA, ligation will not occur. Under the appropriate conditions, even a single base mismatch with the template should be detected. The thermostability of DNA ligase provides the advantages that: 1) it is possible to select the temperature which gives the optimal hybridization stringency for his reactions; and 2) it is possible to amplify the ligation product formed by cycling the temperature between reactions conditions and DNA denaturation conditions, allowing successive cycles of ligation to occur (26, and 23).

The mechanism of T4 DNA ligase initially involves the formation of an enzyme-adenylate complex (A), followed by the transfer of the adenylate from the enzyme to the 5' phosphoryl group of the substrate (B), and finally the formation of the phosphodiester bond between the adjacent DNA substrates (C) (25).

$$E + ATP \text{ ---------> } E - AMP + PPi \quad (A)$$

$$E - AMP + 5' P\text{-}DNA_b \text{ ---------> } AMPP\text{-}DNA_b + E \quad (B)$$

$$AMPP\text{-}DNA_b + DNA_a\text{-}OH3' \text{ ---------> } DNA_a\text{-}P\text{-}DNA_b + AMP \quad (C)$$

Another important point, is the hybridization in solution. Classically, hybridization assays are evaluated by dot blot or Southern blot techniques, in which the amplified target is denatured, immobilized on a nitrocellulose or nylon membrane surface, and then hybridized with the appropriate DNA-labeled probe sequence. These classical hybridization techniques, unfortunately have several limitations, including many manual and labor intensive steps, low sensitivity, and issues related to nonspecific binding of the probe to the membrane surface.

The project of my thesis was based on the development of an alternative method of HIV detection having the following advantages: 1) Speed, 2) Sensitivity, 3) Possibility of Automation. 4) Decrease and possible removal of exponential amplification steps to avoid the problem of carryover.

With the combination of magneSphere streptavidin paramagnetic particles, to enrich the amount of HIV-integrated, and LDR; it was possible to decrease the number of cycles of the PCR to only 15 cycles. Furthermore this method opens future possibilities to eliminate the amplification amplification step of PCR by only using the LDR, after the previous enrichment the samples through the use of magneSphere streptavidin paramagnetic particles.

2. MATERIALS AND METHODS.

2.1 DNA Extraction.

Human genomic DNA and genomic DNA from cell line D35, were extracted with the commercial KIT GENOMIX (Talent srl., Trieste, Italy).

Extraction of Genomic DNA from Blood.

Step 1. Blood lysis/denaturation. Three hundred microliters (µl) of EDTA-anticoagulated blood was mixed with 600 µl of "blood lysis buffer" (8% DTAB [Sigma Chemical, St. Louis, MO], 1.5 M NaCl, 100 mM Tris-HCl, pH 8.6, 50 mM EDTA) in a 2-ml Eppendorf tube (Fremont, CA) and incubated at 68°C for 5 min.

Step 2. Deproteinization. Chloroform (900 µl) was then immediately added, and after mixing by inversion (2-3 times), the sample was centrifuged at 10,000 x g for 2 min in an Eppendorf table centrifuge. A solid plug was present at the layer between the organic and aqueous phase: this allows the pouring of the upper aqueous phase containing the genomic DNA into a new 2-ml Eppendorf tube.

Step 3. DNA precipitation and resuspension. The sample was now ready for the DNA precipitation step: 900 µl of ddH_2O and 100 µl of CTAB (Sigma Chemical) from a 5% solution made up in 0.4M NaCl are added. The final CTAB concentration will thus be 0.3%. After gentle mixing by inversion at room temperature, samples were centrifuged at 10,000 x g in an Eppendorf table centrifuge for 2 min: the DNA-CTAB pellet was resuspended in 300 µl of NaCl 1.2 M to exchange the detergent. After resuspension of the pellet, the DNA was recovered by ethanol precipitation: 750 µl of ethanol were added, and after mixing

by inversion, the samples were centrifuged for 10 min at 10,000 x g (all at room temperature). The supernatant was discarded by pouring, and the pellet was rinsed with 70% ethanol/30% H_2O. After removing the 70% ethanol wash, the DNA was completely dissolved in 300 µl of TE, pH 7.5 (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) within 10 min at 55°C or in a lower volume (26). The concentration was determined spectrophotometrically.

2.2 Cell line.

The cell line D35 (HIV-integrated), was supplied by Dr. Dagaro from Burlo Garofolo's Hospital, Trieste, Italy.

2.3 Synthetic Oligonucleotides.

The Oligonucleotides used in these studies were assembled by the phosphoramidite method, on a Applied Biosystems 394 DNA/RNA synthesizer. These Oligonucleotides are listed below:

HIV-1.

HIV-B1(5'-BIOTIN-CATTTGCATGGCTGCTTGATGTCCCCCCACTGTG-3').

SK-37 (5'-CTTCAGGAACAAATAGGATGGATGACAA-3').

SK-38 (5'-ATAATCCACCTATCCCAGTAGGAGAAAT-3').

SK-39 (5'-TTTGGTCCTTGTCTTATGTCCAGAATGC-3').

SK-19 (5'-ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC-3').

SK-38 Poly A-T* (5'-ATAATCCACCTATCCCAGTAGGAGAAATAATA*-3').

* 3'-Amino-Modifier CPG.

SK-23 (5'- GTCCTTGTCTTATGTCCAGAATG-3').

α-1 Antitrypsin.

A1 (5'-TGTCCACGTGAGCCTTGCTCGAGGCCTGGG-3').

A2 (5'-GAGACTTGGTATTTTGTTCAATCATTAG-3').

A3 (5'-CCCCTCCAGGCCGTGCATAAG-3').

HIV-B1 is complementary to the 1355-1388 region of HIV-1, and was labeled with biotin at the 5', using the synthesizer system. SK-38 and SK-39 amplify a fragment of 114 bp corresponding to the region 1543-1657, and SK-37 and SK-23 amplify a fragment of 139 bp corresponding to the region 1515-1653, in both cases of the conserved *gag* region of HIV-1. SK-19 was used as probe to detect the 1543-1657 amplified fragment, which hybridizes to 1587-1627 region of HIV. SK-38 Poly A-T* was used during the reaction LDR and PCR-LDR, * corresponds to 3'-Amino-Modifier CPG (1-Dimethoxy-trityloxy - 3 - fluorenylmethoxycarbonylamino - propan - 2 - succinoy), used to introduce a primary amine to the 3'-terminus of a target oligonucleotide.

For α1-antitrypsin PCR amplification, the oligonucleotides A1 and A2 were used to amplify from position 9890 to 10109 of genomic sequence. The oligonucleotide A3 was used as probe for the amplified fragment, and hybridizes to the region 11900-1192.

The purification of oligonucleotides was performed: by first deblocking the crude oligonucleotide at 55°C for 6-8 hours. Then, oligonucleotides were precipitated with n-butanol (28), and passed through a H_2O-equilitrated G-25 spun column (5 minutes at 1000 x rpm). The purification of HIV-B1 was performed by first labeling an aliquot of 80 ng of HIV-B1 using the T4 polynucleotide kinase (to see

Probes). Then, 150 µg of cold HIV-B1 were added to hot HIV-B1 (500.000 cpm), and heated at 90°C for 5 min. The mixture was run on a 20% denaturing polyacrylamide gel (8.3 M Urea, 1X TBE), (20 cm x 40 cm x 0.5 mm), at 1800 volts for 11 hours. This gel was exposed on autoradiographic film for 1 hour, and the radioactive band of interest visualized. The band containing the oligonucleotide was sliced in pieces and submerged in 100 mM NH_4Ac and incubated at room temperature for 4 hours. After centrifugation at 2000 x g for 5 min the solution containing the oligo was passed through a H_2O equilibrated G-25 spun column (5 minutes at 1000 x g).

2.4 HIV DNA enriched preparation.

50 µg of human genomic DNA, from healthy individuals, were mixed with 10 ng of DNA from cell line D35, to produce a ratio of 5000 human genoma/1 copy integrated HIV. The mixture (250 µl) was boiled for 5 min to denature DNA. Then 20 ng of HIV-B1 oligonucleotide was added , and SSC (2X final concentration) to give a final volume of 300 µl. Hybridization was carried out for 1 hour at 56°C, and subsequently 50 µl of magneSphere streptavidin paramagnetic particles (MS-PMPs, Promega Inc., Madison, WI) were added. The magnetic beads were previously equilibrated (2 washes with 0.5X SSC, and 2 washes with 2X SSC) and finally resuspended in 600 µl with 2X SSC. Binding reaction streptavidin-biotin was allowed for 30 min at room temperature in a rocker. The complex SA-PMPs_HIV-B1 was captured using the magnetic rack (Promega Inc., Madison, WI), and the supernatant was carefully removed without disturbing the pellet. Magnetic particles were washed with 2X SSC,

and recovered again with the magnetic rack. The washing step was repeated four times. After the last washed, 30 µl of water were added and placed in a 80°C heating block for 3 min. The supernatant containing the DNA, was finally removed.

2.5 PCR Amplifications.

Amplification experiments were performed in a final volume of 50 µl: 1X amplification buffer (10 mM Tris-HCl pH 7.5, 2mM (HIV) or 1.5 mM (α-1 Antitrypsin) $MgCl_2$, 50 mM KCl), 100 µM dNTP's, 25 pmoles SK-38 and SK-39 (for HIV) or, 30 pmoles A1 and A2 (for α-1 Antitrypsin), 2.5 units Amplitaq Polymerase (Perkin Elmer Cetus), and DNA (the amount was dependent on the source. The solution was first heated for 3' at 95°C. The following cycles of amplification were performed HIV (94°C/30sec, 60°C/30sec, 72°C/30sec) 35 cycles, α-1 Antitrypsin (94°C/1min, 60°C/1min, 72°C/1min) 25 cycles. At the end, samples were incubated for 3 min at 72°C. The number of cycles were 25 in the experiments for the quantitative determination of α-1 antitrypsin, while 35 cycles were performed in experiments where visualization on agarose gel was required.

2.6 Probes.

The oligonucleotides were 5' end-labeled with γ^{32}P-ATP to a specific activity of 10^7 cpm/µg of DNA. The reaction mix was: 1X Polynucleotide Buffer (PNK; 0.5 M Tris HCl pH 7.6, 0.1 M $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine, 1 mM EDTA), γ^{32}P-ATP (sp.act.=3000

ci/mmole), 15 units T4 Polynucleotide Kinase, 100 ng oligonucleotides, in 20 µl. Incubation was at 37°C for 1 hour. Unincorporate ^{32}P label was removed, using a H_2O equilibrated spun column with sephadex G-25.

2.7 Southern blotting of amplified sequences.

Samples amplified by PCR were electrophoresed in a 12% polyacrylamide gel with 1X TBE (1X TBE is Tris 0.89 mM ph 8.3, boric acid 89 mM and EDTA 1 mM). The gel was soaked for 10 min. in 0.4M NaOH, and blotted onto Amershan Hybond N+ Nylon membrane (Amershan), for 1 hour using the electroblotting apparatus semi-dry trans-blot (Bio Rad, Richmond, C.A). After transfer, the nylon membranes were neutralized in 2X SSC, and subsequently were cross-linked by exposure to UV light.

2.8 Hybridization.

The prehybridization was carried out by incubation for 2 hours at 68°C in 5X SSPE (1X SSPE is 150 mM NaCl, 10 mM $NaH_2PO_4H_2O$, 1 mM EDTA), 0.5% SDS, and 5X Denhardt's solution. The hybridization was performed using the same solution of prehybridization containing the denatured probe. It was boiled for 5 min., and then put on ice. The temperature of hybridization were: 56°C for α-1 antitrypsin and 60°C for HIV, and were performed overnight. In all cases, the hybridized filters were washes twice with 2X SSPE, 0.1% SDS at room temperature for 10 min.; once with 1X SSPE, 0.1% SDS at 65°C for 15

min; and once with 0.1X SSPE, 0.01% SDS at 65°C for 15 min. The filters were then autoradiographed using Kodak XAR-5 film with a Dupont Cronex intensifying screen at -70°C.

2.9 Quantitative Analysis for the Relative enrichment by PCR.

Four dilutions (500 ng/µl, 250 ng/µl, 100 ng/µl and 10 ng/µl) from genomic DNA were made to analyze the amount of α-1 antitrypsin present in the samples enriched for HIV. The DNA was boiled during the preparation of dilutions, to insure their complete denaturation. After gel electrophoresis and staining with ethidium bromide, the amount of PCR products in each lane was determined in either of two ways: 1) Directly visualizing on the gel, or 2) Cutting and counting in a ß-counter the respective pieces of membrane with the samples, after hybridization. The log amount of radioactivity counted from the membrane, was plotted against the log concentration of the samples (30).

Also, dilutions from D35 cell line were performed, to measure the amount of HIV present in recovered samples, after the treatment with magneSphere streptavidin paramagnetic particles. Four dilutions were made (40 µg/µl, 20 µg/µl, 10 µg/µl and 5 µg/µl) from D35 cell line.

2.10 PCR and LDR.

Sensitivity limit of LDR to analyze HIV. The LDR reaction was in 50 µl containing: 1X ligation buffer, consisting of: 20 mM Tris-HCl, pH 7.6, 100 mM KAc, 10 mM MgAc, 10 mM DTT, 0.6 mM NAD, and 0.1% Triton X-100, 100 pg of plasmid pHIV (pUC18 with a fragment of HIV-1 (771-2324) 1553 bp (cloned and gently supplied by Baralle's Group, ICGEB, Trieste, Italy), 100 ng SK-37, 10 ng ^{32}P labeled primer (SK-38), and 50 units of AMPLIGASE™ thermostable DNA Ligase (EPICENTRE TECHNOLOGIES). 40 cycles at (94°C/30 sec - 60°C/3 min.) were performed .

Two steps PCR-LDR. PCR reaction was carried out in 50 µl containing: 1X PCR buffer, 10 ng of cell line, 250 ng primers 37 and 39, 100 µM dNTP'S, 1.25 units Taq Polymerase. The program used was (94°C/30sec, 60°C/30sec, 72°C/30sec) for 15 cycles.

After PCR, 5 µl of PCR product were added to 50 µl containing: 1X LDR buffer, 10 ng ^{32}P labeled primer (SK-38), 100 ng SK-37, 50 units of DNA Ligase enzyme, and 25 cycles (94°C/30 sec - 60°C/3 min) was performed. The remaining 45 µl of the PCR reaction, were extracted (1X) with phenol/chloroform, precipitated with EtOH plus tRNA, and resuspended in 20 µl, 5 µl were analyzed in the same way of above.

One step PCR-LDR. The combined reactions were performed in 50 µl containing: 1X ligation buffer, 100 µM dNTP'S,10 ng hot SK-38 poly A-T* (the oligonucleotide SK-38 was blocked adding a poly A-T plus 3'-Amino-Modifier CPG, to see synthetic oligonucleotide), 250 ng of SK-23 and SK-37, 2.5 units Taq Polymerase, 80 units of DNA Ligase, and three different targets: a) 10 ng Genomic DNA, b) 20 µl target recuperated with magneSphere streptavidin paramagnetic particles, and c) 100 pg of plasmid pHIV. The program used was: 94°C/30 sec -

55°C/30 sec - 72°C/30 sec, for 15 cycles, and subsequently 94°C/30 sec - 60°C/3 min, for 25 cycles.

Adding stop solution (95% deionized formamide, 20 mM EDTA ph 7.5, 0,05% (w/v) xylene cyanol FF, 0.05% (w/v) bromophenol blue), the samples were run in 15% polyacrylamide gel (7M Urea in a buffer of 100 mM Tris borate pH 8.9, 1mM EDTA) for 2 hour at 60-W constant power). Gels were fixed in 5% acetic acid, dried and autoradiographed overnight using Kodak XAR-5 film.

3. RESULTS

3.1 Enrichment of HIV-integrated by using magnesphere streptavidin paramagnetic particles (MS-PMPs).

In order to decrease the ratio of genomic DNA with respect to HIV-integrated DNA, and to increase the sensitivity range of detection, HIV-integrated sequences were enriched using MS-PMPs. The protocol (Figure 1) involved mixing 50 µg of human genomic DNA with 10 ng of genomic DNA from a cell line (containing 1 copy number of HIV/haploid genome), which represented the expected signal to background ratio (the predicted 1/5.000). This mixture was hybridized to an excess of oligonucleotide HIV-B1 (position 1355-1388 of HIV-1) biotinylated at its 5 prime end, at 56°C for 1 hour. Due to the fact that streptavidin magnetic particles have a very high capacity and coupling efficiency for biotinylated oligonucleotides, the hybridized mixture was mixed with streptavidin coated magnetic beads to capture the hybrid (HIV-B1-biotinylated)-(HIV-integrated sequence). This hybridized DNA was magnetically removed from the solution, washed and dissolved by incubation in H_2O at 70°C, to liberate the DNA from the paramagnetic particles. The details are described in materials and methods.

The target HIV-integrated enriched has been very useful in the reduction of background noise given by genomic DNA, during the analysis by PCR or LDR. As a first consequence to this, the obtained HIV GENE-PHYS (Gene Enrichment through Positive Hybridization Select) product, allowed an increase in PCR amplification specificity. After PCR amplification of HIV GENE-PHYS product, it has been

possible to directly visualize (on a gel stained with ethidium bromide), the specific product after 35 cycles of PCR. In comparison, with similar samples not treated with MS-PMPs gave no visible PCR product (data not shown).

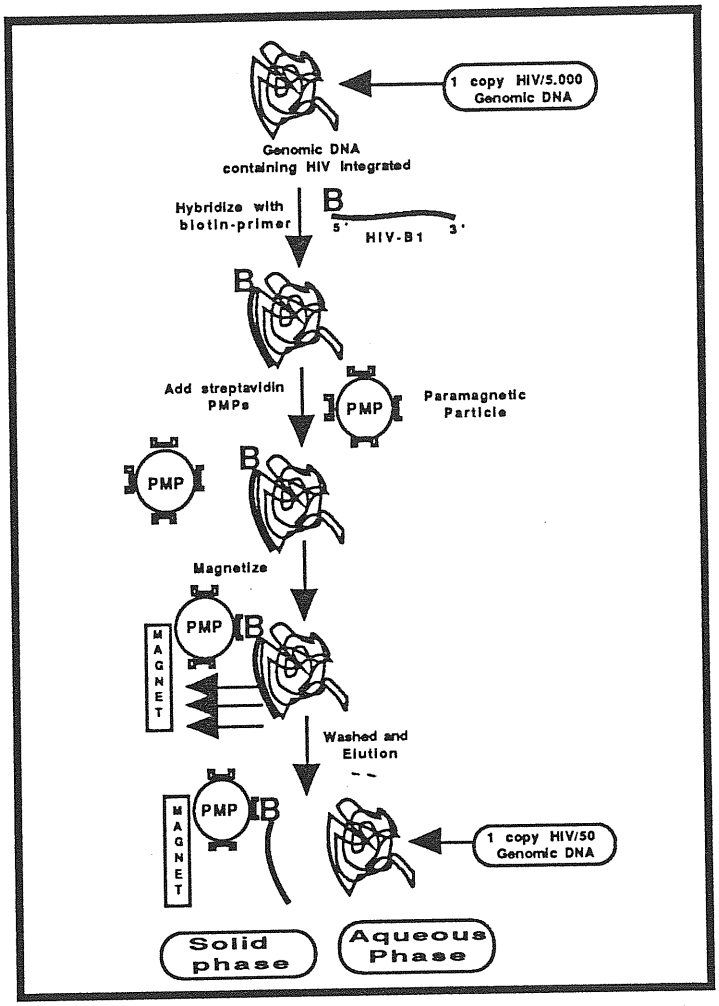

Fig. 1 GENE ENRICHMENT THROUGH POSITIVE HYBRID SELECTION

This method also has the advantage of avoiding contamination from post-amplification products. The oligonucleotide HIV-B1 sequence (1355-1388 bp region) used for enrichment, hybridizes outside of the sequence to be amplified (1543-1657 bp region). Therefore, amplified fragments (carryover), cannot hybridize with the HIV-B1 probe and be a font for contamination.

3.2 Relative quantitation of the levels of α-1 antitrypsin: evaluation of the enrichment for HIV-integrated, after GENE-PHYS preparation.

In order to evaluate the degree of enrichment of HIV-integrated by GENE-PHYS procedure, the level of a gene present 1:1 ratio with respect to the genomic DNA, has been measured and compared by quantitative PCR. In our case, the level of α-1 antitrypsin has been used as reference of comparison between enriched and non-enriched preparations.

Four dilutions of genomic DNA (500 ng, 250 ng, 100 ng and 50 ng), were prepared to be used as standard during the evaluation of α-1 antitrypsin. 10 µl (1/3 of the final preparation) from samples recovered by GENE-PHYS and the respective standard dilutions, were amplified with the primers A1 and A2 for 25 cycles.

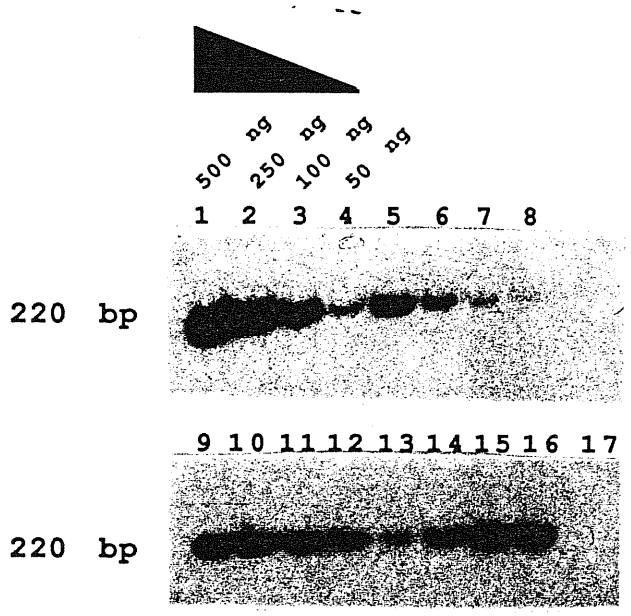

Figure 2. Southern Blot analysis of quantitative PCR amplification of α-1 antitrypsin gene fragment to determine the enrichment of HIV-integrated, after GENE-PHYS procedure. Lanes 1 to 4 represent the standard curve. Lanes 5 to 16 are PCR product of different and independent GENE-PHYS preparations. Line 17 is the negative control.

After PCR amplification, 20 µl of PCR product was run in a polyacrylamide gel, which was electroblotted onto a nylon membrane and hybridized with the labeled oligonucleotide A3.

A visual comparison of the respective band intensities recovered by GENE-PHYS, shows a relative intensity between 50 ng and 100 ng with respect to the bands of the standard curve (Figure 2).

Subsequently, the Southern blot membrane was cut and counted in a ß-counter to determine the counts per minute (cpm). Figure 3 shows the standard curve constructed with the log cpm, obtained from the dilutions, versus the log of the concentration of these dilutions. By intercepting the curve of this graphic, with the cpm from the respective samples recovered by GENE-PHYS, it has been possible to calculate the relative concentration of α-1 antitrypsin present in these samples.

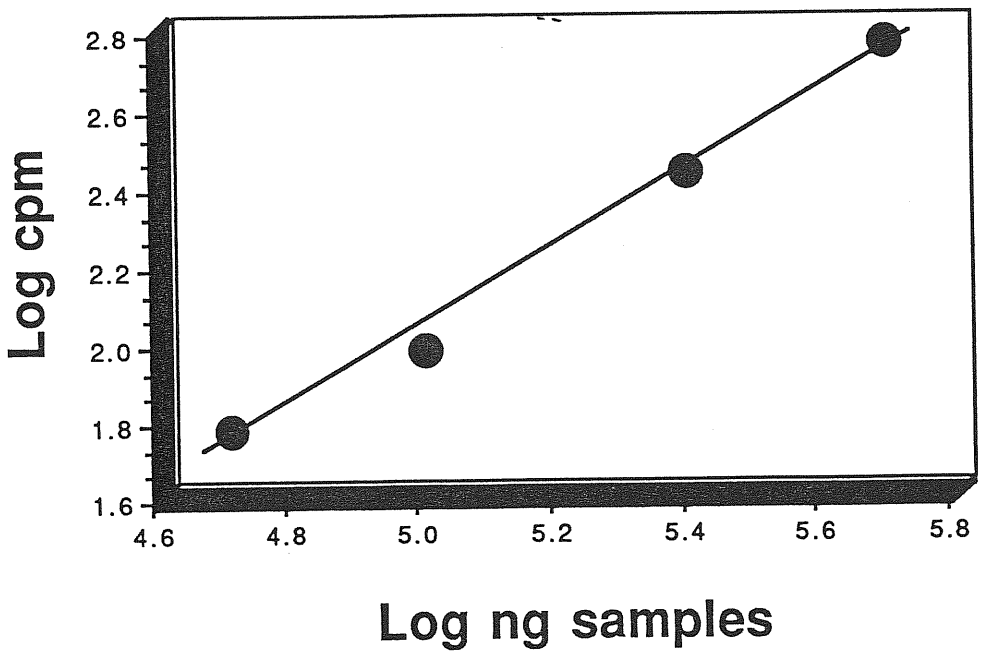

Figure 3. Plot of quantitative relative PCR. Standard curve constructed with four dilutions (500 ng, 250 ng, 100 ng, 50 ng) from genomic DNA, using the respective primers to amplify α-1 antitrypsin.

Since the results obtained from 10 µl of samples recovered by GENE-PHYS, correspond to those obtained from 50-100 ng of total genomic DNA, it can be estimated that 30 µl of samples (total amount after enrichment by GENE-PHYS), correspond to 150-300 ng of genomic DNA. This value correspond to 0.3-0.6% of the initial amount of DNA. Therefore, the enrichment factor of HIV-integrated after GENE-PHYS procedure, has been about 150X to 300X, and the final ratio shifts from 1 HIV/5,000 genomic DNA to 1 HIV/50 genomic DNA.

3.3 Relative quantitation of the level of HIV-integrated after GENE-PHYS procedure.

The enriched samples were then analyzed, by quantitative PCR, to evaluate the amount of HIV-integrated recovered after the GENE-PHYS procedure.

Four dilutions (20 ng, 10 ng, 5 ng and 2.5 ng) from the cell line D35 were prepared, to be used as the standard curve. 30 µl from samples treated by GENE-PHYS procedure, and the respective standard were amplified with the primers SK-38 and SK-39 for 35 cycles. 20 µl of PCR product were run in a polyacrylamide gel, and electroblotted onto nylon membrane. The membrane was hybridized with the labeled oligonucleotide SK-19. Figure 4 shows the autoradiography after Southern blot analysis.

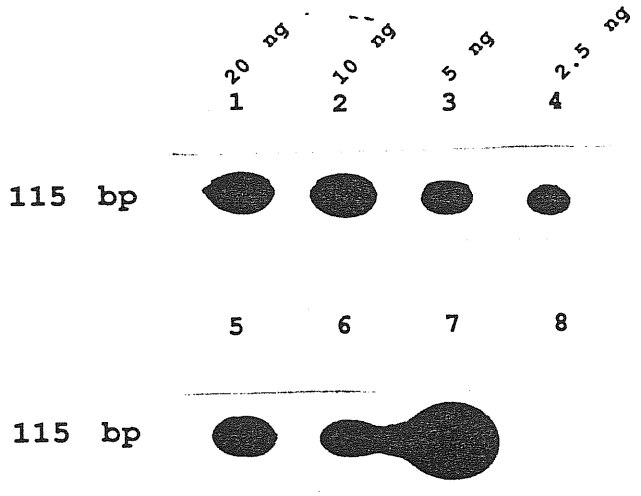

Figure 4. Souther Blot analysis of quantitative PCR amplification of HIV fragment to determine the final amount recovered, after enrichment process. Lanes 1 to 4 are the standard curve from cell line D35 (containing 1 copy number of HIV/haploid genome). Lanes 5 and 6 are two different samples after GENE-PHYS procedure. Lane 7 is a positive control using pHIV (100 ng). Lane 8 is the negative control with water.

The results determined by inspection of the autoradiography, show a relative amount of HIV from enriched samples, that is similar to 10 ng of total genomic DNA from the cell line D35, present before the enrichment by using GENE-PHYS procedure. This result shows that the recovery of HIV during GENE-PHYS is quantitative, and confirmed the high efficacy and sensitivity by the use of MS-PMPs.

In conclusion, the use of GENE-PHYS preparation has allowed an 100 X enrichment of HIV present in a starting ratio of 1:5,000, with a concomitant reduction of background that has allowed direct visualization of the relative PCR.

3.4 PCR-LDR assays.

In order to evaluate the sensitivity of LDR, 100 pg of pHIV was amplified by LDR. Forty cycles of LDR were needed to obtain a sufficiently detectable signal. Figure 5 shows the amplified LDR product, using the primers SK-37 and SK-38 labeled at the 5 prime end.

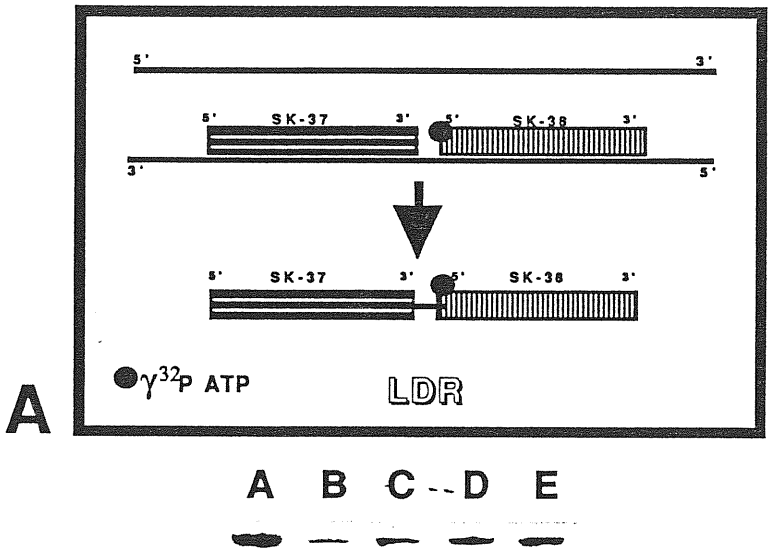

Figure 4. LDR ASSAY. (A) Schematic representation of LDR amplification, using the primers SK-37 and SK-38 labeled at the 5 prime end. (B) Autoradiography showing the result of 5 diferent reactions with 100 pg of pHIV, after 40 cycles of LDR.

However, since LDR is a linear method of amplification, consequently of lower sensitivity, it has not been possible to detect HIV sequences from GENE-PHYS preparation by using the LDR technique only.

I thus decided to increase the initial target by performing fewer cycles of PCR, to be used subsequently as LDR substrate (Figure 6).

Two different tubes were used during the PCR and LDR reaction. Initially, 10 ng of cell line D35 were amplified with SK-37 and SK-38 primers during 15 cycles of PCR. Subsequently, 5 µl of PCR product were amplified by LDR for 25 cycles, with the primers SK-37 and SK-38 labeled at its 5 prime end.

Theoretically, only three products should be obtained after the PCR-LDR assay (Figure 6, A). The first represent the LDR amplified product by using the primers SK-37 and SK-38 labeled at its 5 prime end. The second product represents the PCR amplified product by using the primer SK-39 and the LDR product. The third product represent the PCR amplified product by using the primers SK-39 and SK-38 labeled at its 5 prime end. The remaining products observed are thus nonspecific products.

In this experiment, it was not possible to avoid the action of the enzyme taq polymerase, which contributes to the formation of PCR specific and nonspecific product. These products could compete with the LDR product in the use of the primer SK-38 labeled, thus decreasing the sensitivity of LDR. Other problems found were: (i) it is necessary to open the first tube to remove the aliquot for the subsequent LDR amplification, which increases the possibility of contamination by carryover, (ii) since the Tm of the three primers used was the same, one of them (SK-37) being used in both PCR and

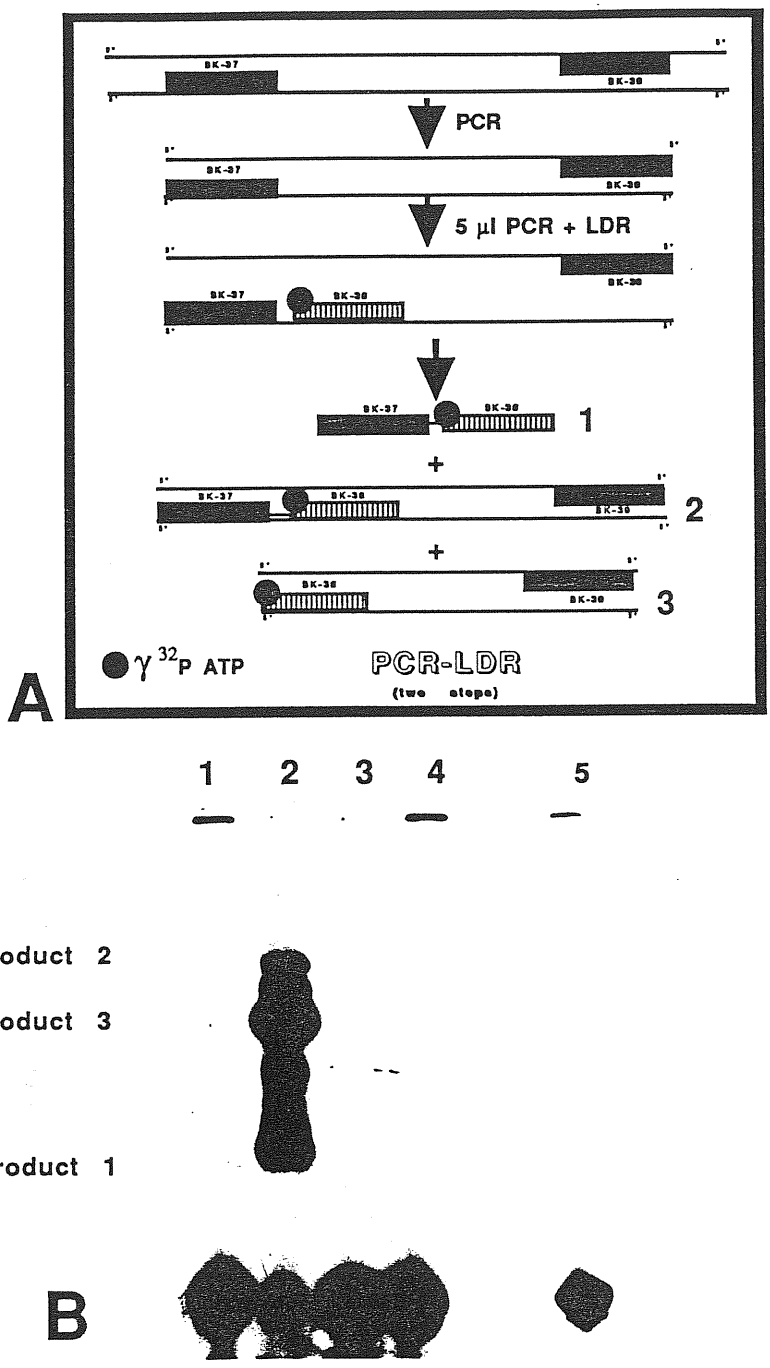

Figure 6. PCR-LDR ASSAY in two steps. (A) Schematic representation showing at less three possible products after PCR-LDR assay (15 cycles PCR and 25 cycles LDR). In this experiment one tube was used for each reaction. (B) Autoradiography showing: Lane 1 and 5, are negative controls; Lane 2 using 5 µl from PCR product; Lane 3 using 5 µl PCR product purified with phenol/chloroform, Lane 4 1 ng pHIV as positive control.

LDR techniques, and the annealing temperature used during the PCR and LDR was also the same (60°C), annealing of the three primers occurred during both reactions, (iii) since the primer SK-38 was not blocked, it was also used for amplification, giving different products:

one of them was the product amplified by using the LDR product and SK-39 as primers, and the other was the product amplified between SK-38 and SK-39.

Thus I wanted to evaluate (Figure 7), the possibility to perform both reactions (PCR and LDR), in the same tube at the same time. Initially the buffers of the PCR and LDR were tested separately, to determine the best buffer to be used during the combined reactions, combining both enzymes (taq and ligase) in the same tube. The best result was obtained, using the LDR buffer (data not shown).

In order to avoid the action of the polymerase on the primer SK-38, this oligonucleotide was blocked at the 3' end in two different ways: 1) a tail poly A-T was added at its 3 prime end during the synthesis, and 2) a 3'-Amino-Modifier CPG (1-Dimethoxytrityloxy-3-fluorenylmethoxy-carbonylamino-propan-2-succinoy), was chemically introduced during synthesis, after the poly A-T. This group introduces a primary amine at the 3'-terminus of the target oligonucleotide, thus avoiding its use as a primer for the polymerase. Furthermore, the PCR specific primer (SK-23), was made shorter, thus decreasing its Tm.

The PCR was performed at 55°C to allow the action of the primers SK-37 and SK-23 for 15 cycles. This was followed by 25 cycles of LDR at 60°C, to only allow the action of SK-37 and SK-38 poly A-T*, avoiding the attachment of SK-23 which has a lower Tm.

Lane 2 (10 ng of D35 DNA + taq + ligase), shows three product: the first represent the LDR amplified product (principal product), by using the primers SK-37 and SK-38 poly A-T* labeled at the 5 prime end; the second represent the PCR amplified product by using the primer SK-23 and SK-38 poly A-T* labeled at the 5 prime end, and the third product represent the PCR amplified product by using the primer SK-23 and the LDR product. In comparison with the LDR product, the

signal produced for the products 2 and 3 was lower. The explanation of PCR product formation, using as primers SK-38 poly A-T* or the LDR product, could be that the polymerase has 3 prime exonuclease

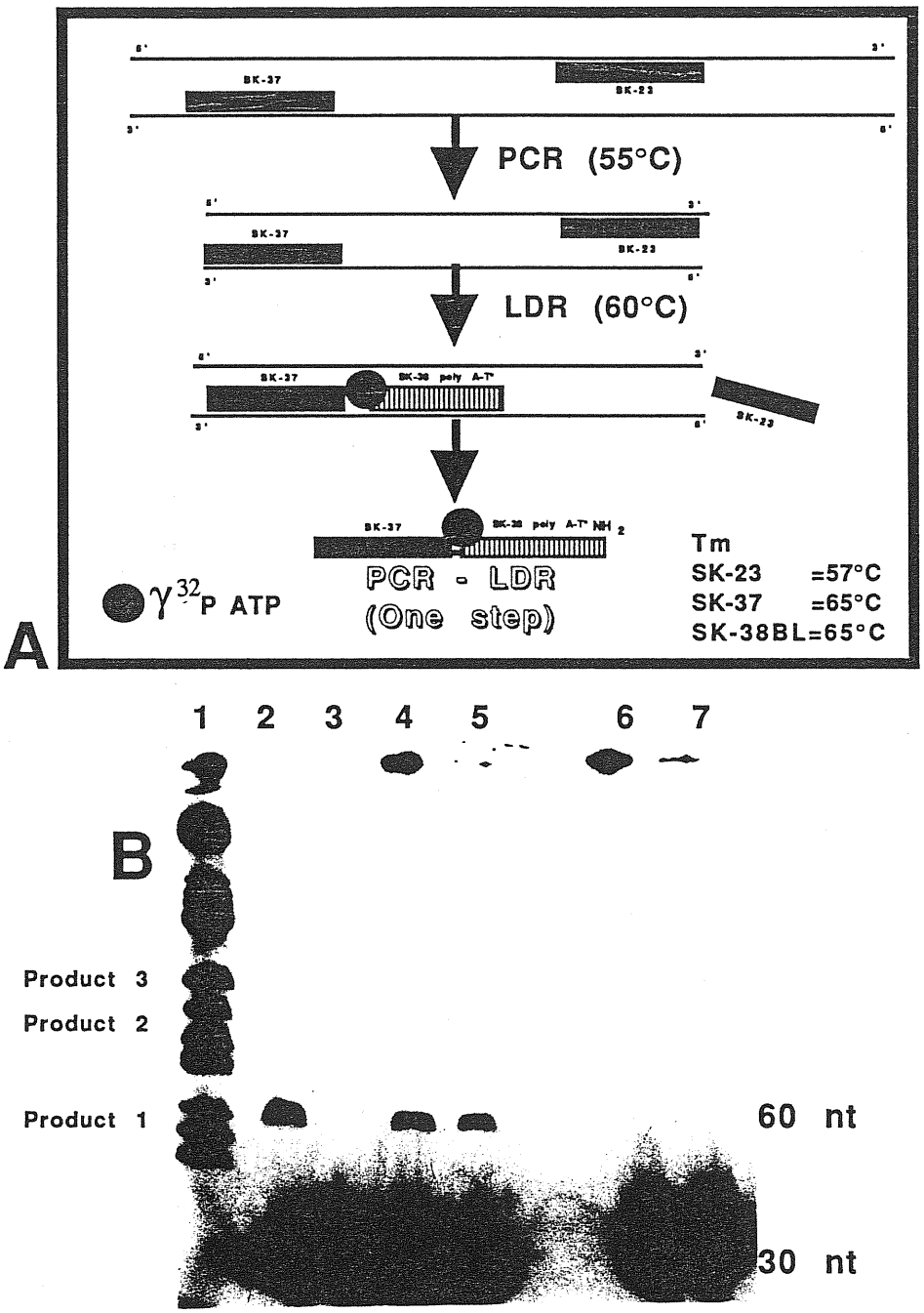

Figure 7. PCR-LDR ASSAY in one step. (A) Schematic representation showing the protocol used to increase only the LDR product. (B) Autoradiography showing: Lane 1: M.W. marker Boehringer VI; Lane 2: 10 ng D35 + ligase + taq; Lane 3: 10 ng D35 +taq; Lane 4 100 pg pHIV + ligase; Lane 5: GENE-PHYS preparation + ligase + taq; Lane 6: 10 ng genomic DNA + ligase + taq; Lane 7: Negative control. (PCR = 15 cycles, LDR = 25 cycles).

activity, and thus making available the blocked primer (SK-38 poly A-T*). Lane 3 (10 ng of D35 DNA + taq) shows the PCR amplified product produced by using the primers SK-38 poly A-T* and SK-23. Lane 4 (100 pg pHIV + ligase), shows a positive control: the LDR amplified product formed by using the primers SK-37 and SK-38 poly A-T*. Lane 5 (GENE-PHYS preparation + ligase + taq) shows the LDR amplified product formed by using the primers SK-37 and SK-38 poly A-T*, and the PCR amplified product formed by using the primer SK-38 poly A-T* and SK-23. Lane 6 (10 ng genomic DNA + ligase + taq) represent a negative control. Lane 7 is a negative control without genomic DNA.

In this combined one step PCR-LDR assay it has been possible to detect HIV sequences from GENE-PHYS preparation, using 15 cycles of PCR followed of 25 cycles of LDR. This low number of PCR cycles have been sufficient to increase the initial target and to allow its detection by LDR.

Since the primer SK-23 has a lower Tm, its use during the LDR reaction was avoided using different temperatures of annealing during the reactions. Blocking the primer SK-38 poly A-T* at its 3 prime end, was avoided its use as primer during the PCR reaction.

The advantages found using this protocol are the following: (i) the PCR-LDR reaction can be made in only one tube, avoiding contamination from the samples by carryover, (ii) the specificity of the reaction is increased, obtaining the LDR product as the principal product, and (iii) the sensitivity range to detect HIV-integrated from GENE-PHYS preparations, was increased.

This experiment proves that both reactions (PCR and LDR) can be performed under controlled conditions, in the same tube.

4. DISCUSSION

Many alternatives have been proposed for the detection of HIV, most of them being based on amplification by PCR, and usually detected by Southern blotting hybridization with a labeled probe.

In this work I have described and characterized a method, which allows to increase the degree of detection of HIV-integrated present in the genomic DNA, through both the enrichment given for the use of MS-PMPs and the amplification performed by combining PCR and LDR.

The enrichment obtained after GENE-PHYS preparation, has allowed to decrease the initial ratio of HIV-integrated from 1/5,000 to 1/50, which has facilitated the detection by PCR-LDR assay. In fact, 35 cycles of PCR were sufficient to directly visualize, on agarose gel, the HIV amplified, which could not be performed in samples without previous treatment with MS-PMPs. This result can be considered important by itself since Southern blotting is avoided. However, at this point, 35 cycles of PCR are still necessary to detect the band; moreover, the possibility of contamination by carryover is always present.

Many protocols used in the diagnosis of several diseases are based on the use of PCR amplification, since PCR increases the amount of target to be analyzed thus allowing easy detection. At the same time, the carryover produced from previous amplifications, has been considered a serious problem during the development of screening programs. Sterilization procedures have been devised to overcome the problem of carryover, but their use do not completely insure the total degradation of possible fonts of contamination. In

this work, I have proposed the possibility of using a non-exponential method to avoid contamination in subsequent analysis. In fact, the LDR product cannot be considered a potential carryover, because the product formed cannot be used as target for the primers during a new LDR reaction.

Since the LDR, being a linear and not exponential amplification process has a lower sensitivity, the HIV-integrated from GENE-PHYS preparation was not detected by this method. Thus I have introduced few cycles of PCR to increase the initial target. Fifteen cycles of PCR were sufficient to increase the target for LDR reaction, which decreases the possibility of contamination by carryover. The use of specially designed oligos, aided to decrease the number of sub-products, thus increasing the specificity of LDR.

The perspectives for the future are showed in figure 8, which comprises: 1) Enrichment of HIV-integrated by using GENE-PHYS procedure, which increases the target 100-fold (from 1/5,000 to 1/50). 2) Amplification of GENE-PHYS preparations during 40 cycles by LDR, thus theoretically increasing the target 40-fold 3) Hybridization in solution by using a synthetic RNA probe labeled with digoxigenin, increasing the kinetic of hybridization and the sensitivity. At the end, the non-hybridized probe is digested with RNases to decrease the background (RNase Protection Assay). 4) Finally the RNA/DNA protected hybrid can be detected by using a chemiluminescent reaction that is least 10-fold more sensitive in comparison with radioactive methods.

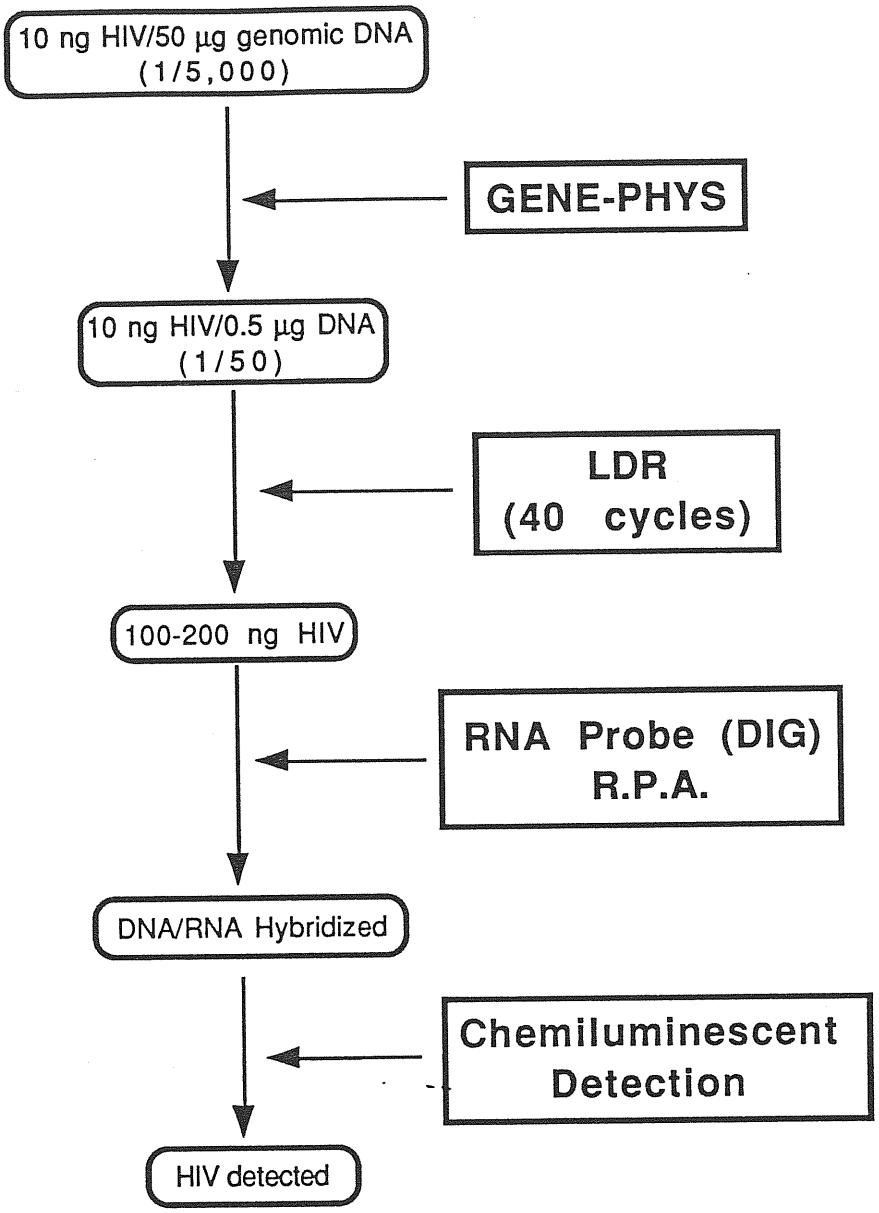

Figure 8. Schematic representation of the perspectives for the future.

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