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Evaluation of Several Parameters of *in situ* Polymerase Chain Reaction (ISPCR) to Reduce the Leakage of Amplificants from Cells

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Proviral DNAs from HIV-1-infected CD4+ T cells (Molt/LAV cells) were amplified and detected in infected individual cells using polymerase chain reaction and *in situ* hybridization. In this *in situ* PCR, three parameters were considered to achieve effective amplification and retention of amplificants inside the cells by making high molecular weight PCR products intracellularly, forming agarose matrix against the cells, and maintaining the appropriate PCR temperature profile. Over the cycles of amplification, tailed primers with complementary overhanging sequences at their 5' sides manufactured high molecular weight products by using short primary products as a repeating unit. Agarose matrix could prevent the diffusion of the amplificants from the cells. Use of Thermanox coverslip inside the PCR tube offered target cells a similar temperature profile to that of conventional PCR in solution.

Key words: *in situ* PCR, tailed primers, high molecular weight PCR products, agarose matrix, Thermanox coverslip

Highly specific and sensitive serologic assays require 2 weeks to 6 months for the detection of HIV infection (Lange and Goudsmit, 1987). Upon infection, p24 antigen takes 2 weeks to appear in the serum and lasts 3 to 5 months (Allain *et al.*, 1986). It is therefore difficult to detect viral antigens and HIV particles at early stages of infection. Early diagnosis of HIV infection is therefore essential to monitor seronegative individuals and infants born to infected women for possible transmission of HIV and to institute antiretroviral treatment (Borkowski *et al.*, 1987).

Viral nucleic acid assays have been developed with the advent of nucleic acid probes. Southern blot, Northern blot, and RNA dot-blot were applied for the detection of HIV nucleic acids (Thomas, 1980; Shaw, 1984; Richman *et al.*, 1986; Monroe *et al.*, 1987). *In situ* hybridization (ISH) can also detect the RNA or DNA inside the infected cells or tissue sections. This method is particularly useful in studying slow viruses which have a long incubation period and undetectable levels of viral antigens (Harper *et al.*, 1986). In contrast to the previous assay methods, ISH can precisely localize the specific targets in the individual cells with high sensitivity (Singer and Ward, 1982). Although this method has limitations for detecting the target with low copy number or short size, new technologies such as *in situ* transcription (Tecott *et al.*, 1988) or polymerase chain reaction (PCR) may ultimately alleviate this problem (Mullis and Faloona, 1987).

PCR is a primer-directed method for the enzymatic amplification of specific DNA sequences through successive cycles (Saiki *et al.*, 1988). The final PCR product after 25-35 cycles is a short primary product with both ends defined by the primers. The notion that PCR can amplify HIV DNA in advance to the appearance of antibodies made PCR an excellent tool for early diagnosis of HIV infection (Loche and Mach, 1988). A limitation of PCR with cell-free DNA is the lack of association of amplification with a specific cell type, particularly in the heterogeneous population of a sample. *In situ* PCR (ISPCR) which combines the highly sensitive PCR with ISH, however, amplifies the target sequences in fixed cells or tissues, thereby anatomically localizing the target inside the cell and preserving the cell morphology. ISPCR can be successfully performed both in cell suspension (Haase *et al.*, 1990) and in tissue sections (Staskus *et al.*, 1991) or cells fixed on glass slides (Bagasra *et al.*, 1992).

ISPCR begins first with the amplification of the target sequence inside the cells. Since the cell membrane may serve as a physical barrier, treatment of fixed cells with

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HCl, triton X-100, and/or proteinase K is necessary to obtain the maximum accessibility of PCR reagent to the target sequences across the cell membrane. These treatments, however, should be considered carefully in the context of cell damage, lysis, and/or excessive diffusion of PCR amplificants from cells. Nonspecific amplification and especially the diffusion of the PCR products from the cells may limit the great availability of the ISPCR. For these reasons the efficiency of ISPCR per cycle is very low. To minimize the diffusion of amplificants and retain these inside the cells, several methodologies have been designed and performed (Haase *et al.*, 1990; Nuovo *et al.*, 1991; Chiu *et al.*, 1992). In an effort to develop an adequate ISPCR technique, several parameters such as a pair of primers (tailed primers) with complementary 5'-overhanging sequences and agarose matrix were evaluated for the detection of HIV-1 proviral DNAs in Molt/LAV cells prepared on a Thermanox coverslip inside the PCR tube.

Materials and Methods

Plasmids and their preparations for amplification

The plasmid pBH10 was constructed by the excision of the viral insert (provirus in infected cell line H9/HTLV-III) of BH10 and subcloning into the *Sac*I site of pSP64 (Hahn *et al.*, 1984). This plasmid was isolated from *E. coli* by established procedures (Maniatis *et al.*, 1989). pSP64 is commercially available from Promega Biotech and was used as a negative control.

The concentration of plasmid DNA was determined at OD 260 nm by spectrophotometer. One million copies of circular plasmids were calculated from the plasmid DNA concentration and used for the amplification.

Preparation of chromosomal DNA

Cells were lysed with SDS and proteinase K (IBI, New Haven, CT) at a final concentration of 0.5% and 0.1 mg/ml, respectively in 1×TES (10 mM Tris-Cl, 1 mM EDTA, pH 8.0, 1 mM NaCl) at 65°C for 4 hr. Lysed cells were extracted twice with one volume of phenol and chloroform. Supernatant was mixed with a half volume of ammonium acetate and two volumes of absolute ethanol, and vortexed hard for 5 sec. The white sticky precipitate of DNA was collected, washed with 70% ethanol twice, dried, and resuspended in 1×TE buffer. The concentration of DNA was measured with a spectrophotometer.

Primers and probes

The oligonucleotide primers SK38, SK39, and probes SK19 (Ou *et al.*, 1988), J16, J17, and J18 were synthesized through Gene Assembler Plus oligosynthesizer (Pharmacia, NJ) and purified through NAP-10 columns (Pharmacia, NJ) and Sep-pak C18 cartridges (Millipore, Bedford, MA). Tailed primers TRSK38 and T'SK39, complementary 5'-overhanging version of primers SK38 and SK39, respectively, were also synthesized and purified accordingly. SK19 was radiolabeled with ^{32}P and J16, J17, and J18 with ^{35}S using polynucleotide kinase (New England Biolabs, Beverly, MA) (Chaconas and Van de Sande, 1980). The nucleotide sequences of these primers and probes are as follows:

SK38: 5'-ATAATCCACCTATCCCAGTAGGAGAAAT-3'

SK39: 5'-TTTGGTCCTTGTCTTATGTCCAGAATGC-3'

TRSK38: 5'-ccccaaaccccaaaccccGTAC-SK38-3' (Underlined is the *Rsa*I restriction site)

T'SK39: 5'-ggggtttggggtttgggg-SK39-3' (Small lettered oligonucleotides are complementary 5'-overhanging tail sequences between tailed primers, TRSK38 and T'SK39)

SK19: 5'-ATCCTGGGATTAAATAAAATAGTAAGA-ATGTATAGCCCTAC-3'

J16: 5'-GTAGGGCTATACATTC-3'

J17: 5'-TAAAAGATGGATAATCC-3'

J18: 5'-ACTATTTTATTTAATTCCC-3'

Target region for the amplification (Fig. 1)

A region in HIV-1 (*gag* 1551-1665) was chosen as the target region for the amplification by PCR. This conserved region of *gag* is defined by a primer pair, SK38 and SK39 spanning 115 nucleotides. The probe SK19 which is complementary to the internal sequence (*gag* 1595-1635) was used to identify the PCR amplificants in solution. Probes J16, J17, and J18 were chosen to be complementary to the internal sequences (*gag* 1620-1635,

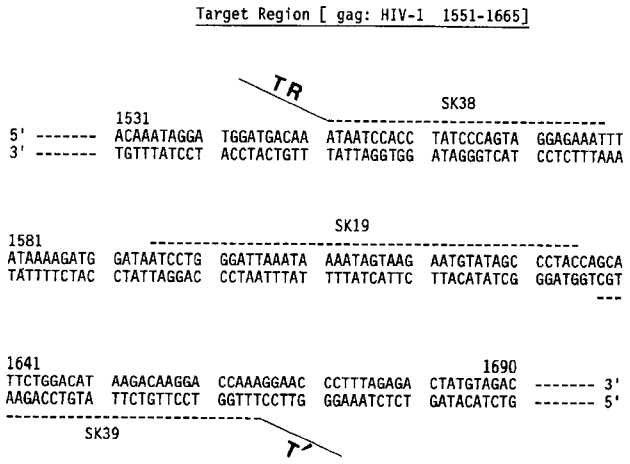

Fig. 1. Target region (HIV-1 *gag* region, 1551-1665). A conserved region of *gag* (nucleotides 1551-1665) is the target for the amplification defined by primers SK38 and SK39. This region was also amplified by a pair of tailed primers, TRSK38 (SK38 primer tailed with GC rich sequence and *Rsa*I enzyme site of GTAC; 5'-CCCCAAACCCCAAAC-CCCGTAC-SK38-3') and T'SK39 (SK39 primer tailed with GC rich sequence complementary to the tail of TRSK38; 5'-GGGGTT-TGGGGTTTGGGG-SK39-3'). In addition to probe SK19, the other oligonucleotide probes J17 (nucleotides 1582-1598), J18 (nucleotides 1600-1617), and J16 (nucleotides 1620-1635) were also used to identify the amplificants.

1582-1598, and 1600-1617, respectively) for ISPCR.

Cell cultures and preparation of the cells for PCR in solution

U937 (a promonocyte cell line), U1.1 (a subclone of U937, post-infected promonocyte with HIV-1) (Folks *et al.*, 1987), and Molt/LAV cells were prepared (Kikukawa *et al.*, 1986) and purchased through the AIDS research and Reference Reagent Program. Upon arrival, the frozen vial was relocated to Biosafety level-3 area and all laboratory manipulations of this cell line until fixation were performed in the same Level-3 area. Cell cultures were propagated according to the instructions.

Cell cultures were rinsed with PBS twice by centrifugation at 1,200 rpm for 5 min and fixed with ethanol: glacial acetic acid (3:1) for 5 min. Cells were washed with 100% ethanol and kept in 70% ethanol at 4°C until further use.

Cells were collected from 70% ethanol by centrifugation, rinsed with PBS, and rehydrated in PBS at room temperature for 20 min. The concentration of cells was measured with a hematocytometer using trypan blue. Cells in PBS were collected, rinsed with double distilled water (ddH_2O) for PCR and resuspended in ddw to make 2×10^6 cells/ml (4 volumes of 5×10^5 cells/ml). One volume of cells in ddH_2O was placed in a 0.5 ml microcentrifuge tube and mixed with other reagents for the PCR.

PCR in solution

PCR condition

PCR conditions using plasmids or cells in a 0.5 ml microcentrifuge tube were optimized with primers (0.5 μM each), dNTPs (250 μM each; Pharmacia, Piscataway, NJ), Taq polymerase (5 units in 50 μl reaction; Perkin Elmer, Norwalk, CT), and PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, and 2.5 mM $MgCl_2$). Reaction mixture (50 μl) was overlaid with 50 μl of light mineral oil (Sigma Co., St. Louis, MO) in the 0.5 ml microcentrifuge tube.

DNA Thermal Cycler Protocol

A DNA Thermal cycler (Perkin Elmer, Norwalk, CT) was used for the amplification. Samples without Taq enzyme were denatured at 100°C for 3 min and switched to 94°C with addition of Taq. Thermal cycling was as follows: 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min. After certain cycles, samples were withdrawn from the tube and analyzed by agarose gel electrophoresis.

Gel electrophoresis, Southern transfer, Hybridization, and Autoradiography

Amplified products (15 μl) were analyzed on an agarose gel of 1% NuSieve and 1% SeaKem GTG agarose (FMC Bioproducts, Rockland, ME) under UV light after running at 100 volts for 1-2 h and staining with ethidium bromide.

Amplified DNAs in the gel were blotted onto a nitrocellulose membrane as described (Maniatis *et al.*, 1989). Prehybridization and hybridization were carried out at 58°C for 3 h and overnight, respectively. Blots were washed three times at room temperature. The first wash was done with 2×SSC, 0.1% SDS for 10 min, the second wash with 1×SSC, 0.1% SDS for 5 min, and the third wash with 0.1×SSC for 1 min.

The blots were exposed to Kodak Diagnostic Film X-OMAT (Kodak, Rochester, NY) at -70°C. After exposure, the films were developed in a Konica QX-60A automatic x-ray film processor (Konica, Tokyo, Japan)

ISPCR of cells on Thermanox coverslips (T.Cs) in tube

Preparation of cells on T.Cs

Cells were rinsed, counted, and laid over the T.Cs (Nunc, Naperville, IL) in a circle. Prior to the running of PCR, a T.C with a circle of cells was cut into the inner contour size ("oval shape") of the 0.5 ml microcentrifuge tube and immersed into the PCR solution or the agarose-PCR mixture.

PCR condition

PCR reagents were prepared the same as those for PCR in solution. The agarose-PCR buffer mixture was prepared by 1:1 mixture of prewarmed 5% SK GTG agarose and 2×PCR solution. One hundred microliters of PCR solution or agarose-PCR buffer mixture was inserted with a cut-T.C and overlaid with 50 μl of mineral oil. The Cycler was programmed to 50 cycles of 94°C for 1 min (first cycle for 5 min), 55°C for 2 min, and 72°C for 1 min, and terminated to 25°C. The oil was then removed, and the T.Cs were pulled out of the tube. The T.C from the PCR solution was subject to post-PCR fixation in 4% paraformaldehyde for 1 h. The T.C with the agarose-PCR buffer mixture was also fixed for each 30 min before and after the removal of agarose-PCR buffer mixture. The T.Cs were then washed with PBS buffer for 5 min and dehydrated with 70% ethanol. Dehydrated T.Cs were attached to the glass slide with nail polish, and these were treated as a unit.

ISH and Autoradiography

Amplified DNAs were denatured and neutralized by immersing the slides in 0.5 M NaOH/1.3 M NaCl and 1.0 M Tris-Cl/1.5 M NaCl, respectively for 10 min each. Slides were dehydrated with 70% ethanol and air-dried.

Hybridization solution (1 μg/μl salmon sperm DNA, 1 μg/μl of *E. coli* tRNA, 4×SSC, 0.33% SDS, 5% dextran sulfate, 1.1×Denhardts solution and probes) was heated to 95°C for 7 min, cooled on ice for 5 min, and added with DTT (dithiothreitol) to a final concentration of 33 mM. The slides were overlaid with 60 μl of hybridization solution on each, covered with siliconized 22×50 mm coverslip, sealed with rubber cement (Dennison Co., Framingham, MA), placed into the humidified slide chamber, and incu-

bated at 26°C overnight. At room temperature, the coverslips were removed in 4×SSC and the slides were washed twice in 1×SSC for 20 min each. The washed slides were treated twice for 3 min, first with 70% ethanol/0.3 M ammonium acetate and next with 95% ethanol/0.3 M ammonium acetate, and were air-dried. NTB-2 emulsion (IBI, New Haven, CT) was prewarmed to 43°C and coated onto the slides. The slides were air-dried in darkness for 2 h and exposed at 4°C for 3-4 days in a light-tight slide box. The slides were developed in darkness in D-19 (IBI, New Haven, CT) for 5 min, 2% acetic acid for 3 min, and rapid fixer for 5 min and rinsed in ddw for 15 min. The cells on the slide were counterstained with Wright-Giemsa stains for 15 min, rinsed in running water, air-dried, mounted with Pro-Texx mounting medium (American Scientific Products, McGaw Park, IL), and observed under a light microscope.

Results and Discussion

The main reason for the use of tailed primers is to create high molecular weight products, in which it is hard for these bulky products to leak out of the cells. Over the cycles of amplification, short primary products of PCR can serve as primers to the target or much more frequently bind to one another through the annealing between two complementary tails, thereby providing a 3'-OH site for polymerization by Taq enzyme. The annealing can be stabilized by 67% of high GC content and alternating bases of tail sequences. In this case, annealing can happen among 2, 3, or 4 primary products, and so on. The various products made from annealings and polymerizations can also serve as other primers or bind to one another in different combinations. Thus, immense concatemerizations can lead up to high MW secondary products with primary products as repeating units or building blocks. Agarose matrix was also included to prevent the leakage of short primary products in early PCR cycles, thereby providing more building blocks for the high MW products. Use of cuttable Thermanox coverslip inside the PCR tube provides the adequate temperature range to the targets in cells and therefore eliminates the need for glass slides and the specific cycler only for *in situ* PCR use.

PCR in solution

Effect of the primers for the synthesis of high molecular weight (MW) secondary products (Fig. 2)

Optimal PCR conditions for primer pairs specific for HIV-1 were first determined on plasmid pBH10 target DNA. The optimization was performed mainly to achieve not only the highest specificity and sensitivity but also the successful secondary amplification. A PCR buffer condition (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 250 μM dNTPs, and 5 units of Taq enzyme in 50 μl reaction) with 0.5 μM primers was finally selected for the PCR in solution.

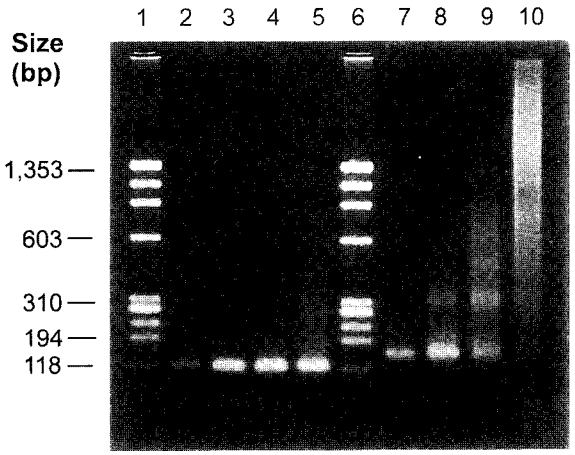

Fig. 2. Effect of complementary tails for the synthesis of high molecular weight secondary products. Tailed and untailed SK38 and SK39 primers were used to amplify the target sequence from pBH10. Amplified products by untailed and tailed primers are shown respectively in lanes 2 and 7 (30 cycles), 3 and 8 (40 cycles), 4 and 9 (50 cycles), and 5 and 10 (60 cycles). A thirteen picogram of pBH10 (1 million copies) was amplified in 100 μl reaction. 15 μl aliquots were analyzed in 2% GTG agarose gel.

A band of expected size using the optimal PCR conditions was observed at 30 cycles (lanes 2 and 7; 105 bp with SK38 and SK39; 155 bp with tailed primers, TRSK38 and T'SK39). The complementary overhanging tails and *Rsa*I site seemed to be included in 155bp product. Through repeating cycles of amplification, untailed primers did not produce high MW secondary products (lanes 3, 4, and 5). Tailed primers in the parallel reaction created the concatemers (secondary products) after 30 cycles and showed the pattern of multimeric bands (concatemerization) at 40 and 50 cycles leading to the high MW secondary products (lanes 8, 9, and 10). The degree of forming concatemers was correlated with the gradual disappearance of the primary product. From these observations, it seemed that the primary products were used as building blocks for secondary products by means of complementary base pairing between tails of primers overhanging at both ends of the primary product as the amount of primers decreased with cycles.

Analysis of the secondary products (Fig. 3)

The target sequence (GTAC) of *Rsa*I enzyme in TRSK38 was incorporated into the primary product along with the complementary sequences (18 bp). Digestion of the 30 cycle product with *Rsa*I (lane 4) shortened the primary product (155 bp) and primer dimers (approximately 96 bp; a band below the primary product band) to the 135 bp and 76 bp, respectively, in lane 5. Involvement of either primer dimer or individual primer itself in the formation of the secondary product and high MW product is shown

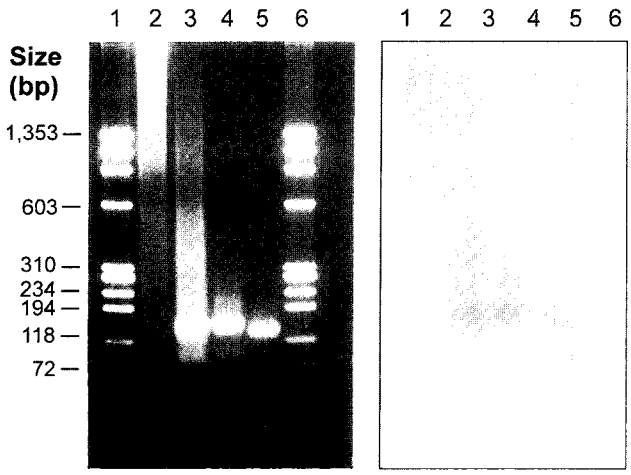

Fig. 3. Analysis of the secondary products by *Rsa*I digestion. (Left) Undigested and digested products of the 30 and 60 cycle reactions. 15 μl aliquots of the 60 and 30 cycle reactions (undigested) were loaded in lanes 2 and 4, respectively. The same amount of the reaction was digested with *Rsa*I (lanes 3 and 5, respectively). (Right) Detection of target sequences by Southern blotting. After photography, the agarose gel shown on the left was Southern transferred for the analysis of the target sequences using ^{32}P-labeled SK19.

in lanes 2 and 3. Digestion of the 60 cycle product with *Rsa*I showed the disappearance of the high MW product and left the incomplete digestion smear as well as 3 bands suggesting the random binding of the various combinations of two tailed primers to the primary and the secondary product during the amplification. Undigested 30 cycle product (lane 4) contained the primary product and the primer dimer. Two bands from lane 5 revealed the detachment of 5'-side of the tailed TRSK38 at *Rsa*I target site (GTAC) from the primary product and from the primer dimers. Southern transfer analysis showed that the bands or smears below 118 bp size marker did not hybridize with the internal probe SK19. From *Rsa*I digestion pattern and Southern analysis, target sequences (primary product) are known to be incorporated into high MW secondary products. It would be therefore possible to localize the amplified product at its site of synthesis inside the cells by applying these conditions *in situ*.

Amplification of different targets (Fig. 4)

Different positive and negative targets were used for the amplification. As positive targets, plasmid pBH10 (lanes 2 and 10), extracted chromosomal DNA of U1.1 cells (lanes 4 and 12), and ethanol: acetic acid (3:1)-fixed U1.1 cells (lanes 6 and 14) showed a band of short primary products at 30 cycles and produced high MW secondary products at 60 cycles. From the fact that the chromosomal DNA of U1.1 cell contains about 2 HIV-1 proviral copies (Folks *et al.*, 1987) and the supernatant of fixed U1.1 cell amplification contained amplificants of target sequences, it was evident that the short products were leaked from the cells during the reactions. The possibility of contamination of any PCR reagents with target sequences was eliminated by the reaction including all the reagents except for any targets (lanes 8 and 16). Presence of any target sequences in the amplificants was verified by Southern blot using internal probe SK19 (right panel).

Plasmid pSP64 (lanes 3 and 11), extracted chromosomal DNA of U937 cells (lanes 5 and 13), and 3:1 ethanol : acetic acid fixed-U937 cells (lanes 7 and 15) as negative targets showed no bands at 30 cycles and produced secondary products at 60 cycles. The result of Southern blot on the right panel shows that the secondary products formed from negative targets at 60 cycles were not derived from any target sequences but from nonspecific concatemerization of tailed primers such as the phe-

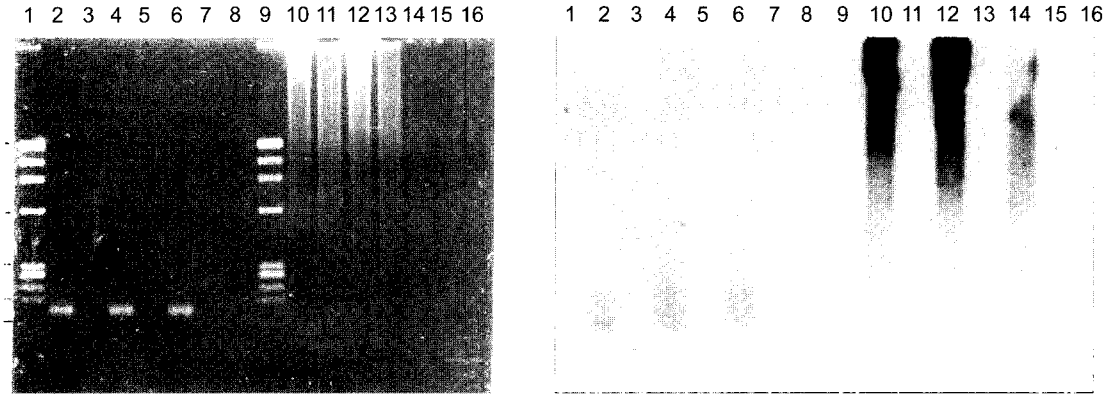

Fig. 4. PCR reactions using positive and negative targets. (Left) An aliquot (15 μl) of each PCR reaction after 30 cycle was loaded in lanes 2 (pBH10 as a positive target plasmid DNA), 3 (pSP64 as a negative target plasmid DNA), 4 (extracted DNA from U1/HIV-1 cells as a positive target chromosomal DNA), 5 (extracted DNA from U937 cells as a negative target chromosomal DNA), 6 (fixed U1/HIV-1 cells as positive target cells), 7 (fixed U937 cells as negative target cells), and 8 (neither DNA or cells). The same amount of the respective 60 cycle PCR reaction was loaded in lanes 10 through 16. (Right) Detection of target sequences. The agarose gel shown on the left was analyzed by Southern blot for the presence of target sequences using ^{32}P-labeled SK19.

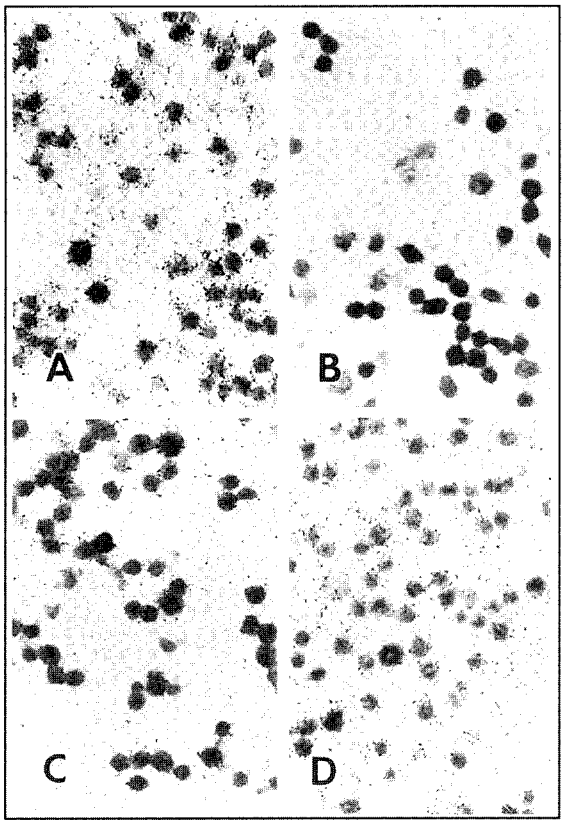

Fig. 5. *In situ* PCR of cells on Thermanox coverslips. Targets in Molt/LAV cells on Thermanox coverslips were amplified in separate tubes under the mixture of PCR buffer and 2.5% SK GTG agarose, PCR buffer solution, tailed or untailed primers. Use of agarose and tailed primers with (panel A) and without (panel B) Taq enzyme were compared to each other. Tailed primers in PCR solution (without agarose; panel C) and untailed primers under the agarose (panel D) were used for the amplification with Taq enzyme. The cycler was programmed for 50 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1 min. After PCR, *in situ* hybridization with ^{35}S-labeled probes of J16, J17, and J18 probes was carried out on the cells fixed in 4% paraformaldehyde. Autoradiography (4 day exposure) was performed upon the coating of the slide with NTB-2. Note some grains can not be seen, are mixed with the dark areas of the cells or appear out of focus in the photograph since the cells are counterstained with Giemsa and grains are positioned at various levels within the emulsion (magnification of ×300).

nomenon shown in reaction without any targets (lanes 8 and 16).

PCR in the presence of agarose

Effect of Agarose on PCR

Under the mixture of PCR buffer and 2.5% SK GTG agarose, PCR was performed on 1 million copies of pBH10 plasmid DNA using 0.5 μM tailed primers in tube. Expected sizes of bands were observed at 30 and 60 cycles (data not shown). From this observation, it was evident that SK GTG agarose is not an inhibitor to PCR and can be used in PCR without significant loss of specificity or sensitivity.

ISPCR of cells on Thermanox coverslips (T.Cs) (Fig. 5)

Molt/LAV cells were selected for the test because this cell line is presumed to contain several tens of HIV-1 proviral copies (Kikukawa *et al.*, 1986). In order to determine the effectiveness of tailed primers and agarose for retention of the amplificants inside the cells, targets in Molt/LAV cells on T.Cs were amplified under the mixture of PCR buffer and 2.5% SK GTG agarose, PCR solution, and tailed primers or untailed primers in tubes. Use of T.C inside the PCR tube also seemed to maintain a PCR temperature profile similar to that of conventional PCR in solution. Dual usage of agarose and tailed primers retained the signals in and around the cells (panel A). Amplification with the tailed primers and omission of Taq enzyme under agarose did not produce amplificants but produced the hybridized signals from the low level of chromosomal target sequences along with background grains (panel B). Under the PCR solution (without agarose) with tailed primers, short products seemed to be leaked out to the PCR solution (panel C). Untailed primers under the agarose seemed to produce the short products, which could be leaked out during the reaction and stuck in between the agarose matrix and the surface of T.Cs (panel D). This interpretation was deduced from the observation of the high level of small sized background grains. Another possible negative control such as uninfected Molt cells was not included here because it did not produce any amplified products of targets in previous studies (Lee, 2001). Clearly enough from these observations, use of tailed primers alone did not produce the satisfactory ISPCR result but dual usage of tailed primers and agarose could be considered as a means for the better retention of the amplificants within the cells containing target DNA.

Acknowledgments

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