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Chromatography in DNA radiolabeling: hands-off automation using a robotic workstation

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Abstract

The experiments described in the present paper were performed in order to determine whether the Biomek-1000 (Beckman Instruments, Fullerton, CA, USA) automated laboratory workstation can be used in a fully automated DNA labeling method followed by automated gravity-driven size exclusion purification of molecular probes. To this aim, we performed random oligodeoxyribonucleotide priming of a HIV-1 LTR probe that was used for molecular hybridization to Southern blotted polymerase chain reaction products. The results obtained demonstrate that the automatically labeled probe can be efficiently purified by automated and gravity-driven Sephadex G-50 chromatography, without any major changes in hybridization property. This robotic methodology can be used in several procedures employing radioisotope labeling.

1. Introduction

Automation plays a pivotal role in the handling of potentially hazardous materials since, for instance, it limits the exposure of laboratory personnel to cancerous and/or toxic substances. Among the most common procedures performed with hazardous materials, the use of radioisotopes in labeling DNA molecular probes is a crucial step in a variety of important applications not only in standard molecular biology assays (such as screening of gene libraries and Southern/Northern blotting analyses) [1], but also in a variety of molecular applications, including molecular diagnosis of genetic diseases, such as aneuploidias in foetal cells by in-situ hybridization with specific chromosome probes [2], and in the identification of very-low-copy number of the HIV-1 viral genome in blood samples [3]. In addition, hybridization steps employing the use of radioactive probes are required in forensic science [4].

In most of these analytical procedures, the laboratory personnel is exposed to β-ray emitters (^{35}S, ^{32}P), mainly during the DNA labeling step. Accordingly, DNA labeling must be performed under safe isotope handling procedures, including the use of rubber gloves and shielding for β-ray protection.

The most used approaches to label DNA at

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high activity are random oligodeoxyribonucleotide priming [5,6], nick-translation [7], 5′-end labeling [8], fill-in [1] and addition of homopolymeric tails to 3′-ends [9]. In all these procedures, nonincorporated radioactive precursors are usually removed by Sephadex G-50 chromatography and fractions quantified by Cerenkov radiation counting [1].

In this paper we determine whether the Biomek-1000 (Beckman Instruments, Fullerton, CA, USA) automated laboratory workstation can be used to perform an hands-off automation of the DNA labeling method followed by automated gravity-driven size exclusion purification.

2. Experimental

2.1. DNA isolation from cells

H938 cell line, derived from the lymphoid T-cell line H9 by transfection with the HIV-1 LTR region, was grown at 37°C in RPMI 1640 medium (GIBCO/Life Technologies, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal calf serum (GIBCO/Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA) in 5% CO_2. The cells were harvested in the late log-phase, washed twice with 0.01 *M* sodium phosphate buffer (pH 7.4) containing 0.15 *M* NaCl (phosphate-buffered saline, PBS), and pelleted by centrifugation at 1000 *g* for 5 min. DNA was then obtained by an automated procedure as elsewhere reported [10].

2.2. Labeling reaction and chromatographic isolation of the molecular probe

The plasmid DNA pTzIIICAT was extracted from bacterial host HB101 by the boiling method [1] and digested with HindIII restriction enzyme. A 2% low-melting point agarose gel was used for separating the digested fragments. The 671 bp fragment was eluted from the gel by QIAEX gel extraction kit (QIAGEN, Chatsworth, CA, USA) and then labeled as described below.

In manual labeling, 50 ng of DNA were labeled using reagents from the random primed DNA labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany) and 5 μl (100 μCi) of [α^{32}P]dCTP (6000 Ci/mmol) to a final volume of 20 μl. The reaction mixture was then incubated 30 min at 37°C.

In automated labeling, 45 μl of the DNA solution (50 ng) were denatured for 5 min at 80°C and then cooled for 3 min at 37°C. The labeling reaction was performed using the Ready-To-Go DNA labeling kit (Pharmacia P-L Biochemicals, Milwaukee, WI, USA). Reaction mixture, containing the preparation (stable at room temperature) vitrified from a buffered solution containing dATP, dGTP, dTTP, Klenow fragment of DNA polymerase I and random oligodeoxyribonucleotides, was obtained by the automated addition of 45 μl of the denatured DNA solution and 5 μl (100 μCi) of [α^{32}P]dCTP (6000 Ci/mmol) to a final volume of 50 μl. The reaction mixture was then incubated 30 min at 37°C.

Nonincorporated nucleotides were removed by size exclusion on NICK-Columns (Pharmacia P-L Biochemicals) using TE (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.5) buffer as eluent. The NICK-columns used in the present study were prepacked disposable chromatographic columns containing Sephadex G-50 fine DNA grade (dry bead diameter: 20–80 μm) in distilled water and 0.15% Kathon CG as preservative, with gel bed dimensions 0.9 × 2.0 cm. Fifteen fractions (4 drops/fraction in the manual method and 150 μl/fraction in the automated method) were collected in 1-ml minitubes. Each fraction was quantified by measuring the Cerenkov radiation. The elution profile was plotted and the fractions corresponding to the first peak were pooled and used in the hybridization experiments.

2.3. Biomek-1000 automated laboratory workstation

Tools and accessories

The program requires P200 and P20 Biomek-1000 pipet tools, appropriate tips, an HCB-1000 thermoelectric control unit and associated heater/cooler unit (Time Logic, Simi Valley, CA,

USA), 96 U-bottom wells PVC microtiter plate, Y-tubes, one tube holder, suitable insert for microfuge, for 12 × 75 mm and 13 × 100 mm tubes, 1-ml minitube holder and matching tubes.

The Y-tube, shown in Fig. 1B, is not commercially available and was built as follows: NICK-column must be installed adjacent to a 12 × 75 mm polystyrene tube by using a 10-mm thick spacer and adhesive tape. The bottom of the chromatographic column is connected by a 25-mm long rubber tube (I.D. = 2 mm) with a hole made at 30 mm from the bottom of the polystyrene tube. The upper 30-mm part of the chromatographic column was cut to allow the Biomek-1000 robotic arm to perform pipetting.

Programming

The configuration of Biomek-1000 is shown in Fig. 1A. P200 and P20 single-tip tools and tips were employed; position 1 was occupied by a 96 1-ml minitube holder; position 2 was occupied by a heater/cooler unit and a 96 U-bottom wells PVC microtiter plate; position 3 was occupied by a 24 well tube holder.

Two subroutines were linked in the method of the DNA labeling reaction and probe purification. The subroutines perform the following processes: (1) DNA labeling reaction and (2) chromatographic steps for probe purification. Each subroutine consists of a number of functions, usually generic pipetting functions. All the generic pipetting functions have the parameters shown in Table 1.

Table 1
Parameters of the generic pipetting functions

Parameter	Condition
Tool	P200 or P20
Source height	bottom
Destination height	top
Dispense rate	1
Type of dispense	to contain: no blowout
Tip touch	yes
Prewet tip	no
Pipetting delay	no
Log	yes

The only exception concerns the chromatographic column loading for which the designated height was 75% from the bottom of a 13 × 100 mm tube.

The program was designed to perform the following steps: (1) pick up a pipet tip and change when appropriate; (2) DNA denaturation for 5 min at 80°C on tray 2; (3) return at 37°C for 3 min; (4) transfer the denatured DNA on tray 3 into the tube containing the vitrified labeling mixture; (5) resuspend the labeling mixture; (6) transfer 5 μl of [α^{32}P]dCTP from tray 1 to tray 3; (7) mix and transfer on tray 2 into suitable position; (8) incubate for 30 min at 37°C; (9) equilibrate the Sephadex G-50 column with 1 ml of TE buffer; (10) discard the flow-through solution on tray 1 into the tube previously containing the [α^{32}P]dCTP; (11) load the labeling reaction solution into the column; (12) transfer 150 μl of TE buffer onto the column; (13) pause for 1 min; (14) transfer the flow-through solution (fraction *n*) into its position on tray 1; (15) repeat steps 12–14 for 14 more times.

2.4. Polymerase chain reaction

Amplification of the HIV-1 LTR region and HLA-DRA gene sequences by the polymerase chain reaction (PCR) [11] was carried out in a 50-μl volume containing 250 ng of genomic DNA, 10 m*M* Tris-HCl pH 8.3, 10 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.5 μ*M* of each primer (the sequences of the primers are summarized in Table 2), 200 μM of each deoxyribonucleotide (dATP, dTTP, dCTP, dGTP). After 5 min of denaturation at 94°C, 2 units of AmpliTaq DNA polymerase, Stoffel fragment (Perkin-Elmer/Cetus, Norwalk, CT, USA) were added to the reaction tube and 30 cycles of amplification were performed (denaturation, 94°C, 1 min; annealing, 60°C, 1 min; extension, 72°C, 1 min).

Polymerase chain reactions were performed on a programmable thermal controller (MJ Research, CA, USA). Size of the amplified products was characterized by electrophoresis on 2.2% NuSieve plus 0.8% SeaKem agarose gel (FMC Bioproducts, Rockland, ME, USA) con-

Table 2
DNA sequences of the primers used in the polymerase chain reactions

Name	DNA sequence
HIV-1 LTR region	
LTR/F	5'-CACACCAGGGCCAGGGGTCAGA-3'
LTR/R1	5'-GCAGTGGGTTCCCTAGCTAG-3'
LTR/R2	5'-CTTGAAGTACTCCGG-3'
HLA-DRA gene	
DRA/R	5'-CTTTGATAGCCCATGATTCCTGA-3'
DRA/F	5'-ACCCTTCCCCTAGCAACAGATGCGTCATCT-3'

taining 0.5 μg/ml of ethidium bromide (Sigma). The gel was photographed on an ultraviolet light box and then blotted to a GeneScreen plus hybridization transfer membrane (Dupont/NEN Research Products, Boston, MA, USA) by the Southern procedure [1] and hybridized with the two [^{32}P]-labeled HIV-1 LTR specific probes.

3. Results

The HIV-1 LTR genomic DNA fragment of 671 bp, obtained after HindIII digestion of plasmid DNA pTzIIICAT, was labeled by the random oligodeoxyribonucleotide primers method [5,6] using both manual and automated protocols. The manual labeling of the HIV-1 DNA fragment was performed by using the Klenow fragment of DNA polymerase I (stored at −20°C in buffer solution) and standard reaction conditions [1]. The labeled probe was separated from nonincorporated [α^{32}P]dCTP by chromatography through a Sephadex G-50 column using continuous-flow elution. This analysis allows to determine both the chromatographic elution profile and the efficiency of incorporation of [α^{32}P]dCTP into the HIV-1 LTR probe.

The automated protocol was performed on a Biomek-1000 automated laboratory workstation (Beckman Instruments, Fullerton, CA, USA). The differences between this fully automated procedure and the manual protocol were the following: (a) a vitrified labeling mixture was used, (b) Sephadex G-50 chromatography was performed by using the Biomek-1000 and (c) chromatographic elution was discontinuous. Fig. 1 shows the tablet layout (panel A) and the Y-tube [10] (panel B) used to perform the fully automated DNA labeling method onto the Biomek-1000 tablet. The chromatographic steps performed by the Biomek-1000 automated laboratory workstation are described in Fig. 2.

The elution profiles obtained from both manually and automated labeling procedures are shown in Fig. 3. This analysis demonstrates that the Sephadex G-50 chromatographic profiles are very similar in both the manual and the automated DNA labeling protocols. In addition, the total cpm obtained by pooling the fractions constituting the first peak were similar (manual method: 7 300 000 total cpm; robotic method: 7 500 000 total cpm).

To demonstrate that the automated DNA labeling procedure closely reproduces the results obtained by the manual procedure, the following experiment was performed. Genomic DNA was automatically extracted from cells [10] of the line H938, which mimics HIV-1 infection of T-lymphocyte in asymptomatic patients [3]. On this genomic DNA we have performed the PCRs for the HIV-1 LTR region and for the HLA-DRA gene (see Fig. 4A for primer location). Three primers (LTR/F, LTR/R1 and LTR/R2) were used for the specific amplification of two different HIV-1 LTR fragments (the 426 bp long LTR/

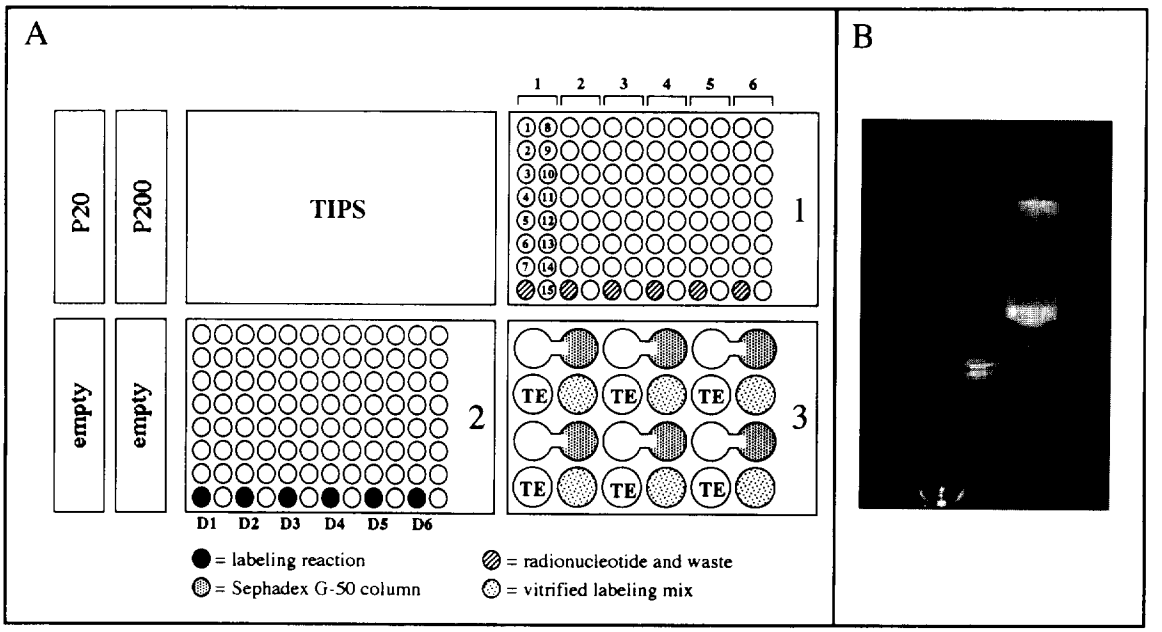

Fig. 1. (A) Biomek-1000 tablet configuration for fully automated DNA radiolabeling and gel filtration procedures. Tray 1: 1–15 = tubes collecting fractions. Tray 2: heater/cooler unit for temperature control of the DNA labeling reactions (D1–D6). Tray 3: chromatography position; the connected circles represent the Y-tubes; TE = chromatographic elution buffer. (B) Y-tube suitable for chromatographic procedures involving the automated recovery of the eluted solutions. The right arm is constituted by the Sephadex G-50 chromatographic column.

F-LTR/R2 and the 232 bp long LTR/F-LTR/R1). As control of the Taq DNA polymerase activity, the human HLA-DRA gene was also amplified by using the DRA/F and DRA/R primers (generating a 262 bp HLA-DRA fragment). The obtained PCR products were electrophoresed in two distinct gels (Fig. 4B), Southern blotted onto nylon membranes and hybridized with the two [^{32}P]-labeled probes specific for the HIV-1 LTR region. The first membrane was hybridized with the probe [^{32}P]-labeled by the manual method (Fig. 4C, left panel), the second membrane was hybridized with the probe labeled by automated protocol (Fig. 4C, right panel). The results obtained clearly demonstrate (a) that only specific amplification products hybridized with both probes, and (b) that hybridization backgrounds were similar.

Taken together, these data demonstrate that the automated procedure described does not change the labeling efficiency and the molecular properties of the labeled probes.

4. Discussion

In recent years, automation was found very useful in several biomedical applications, particularly in performing a number of tedious procedures employed in molecular biology [12–16] and in ELISA-based diagnostic assays [17]. The Y-tube [10,18] allows the automation in chromatography and was originally designed and tested to be used with the Biomek-1000 automated laboratory workstation to perform automated DNA isolation from in-vitro cultured cells and biological fluids, including blood samples. This procedure could help in avoiding the risks of infection of laboratory personnel by pathogenic agents. Elsewhere we published that

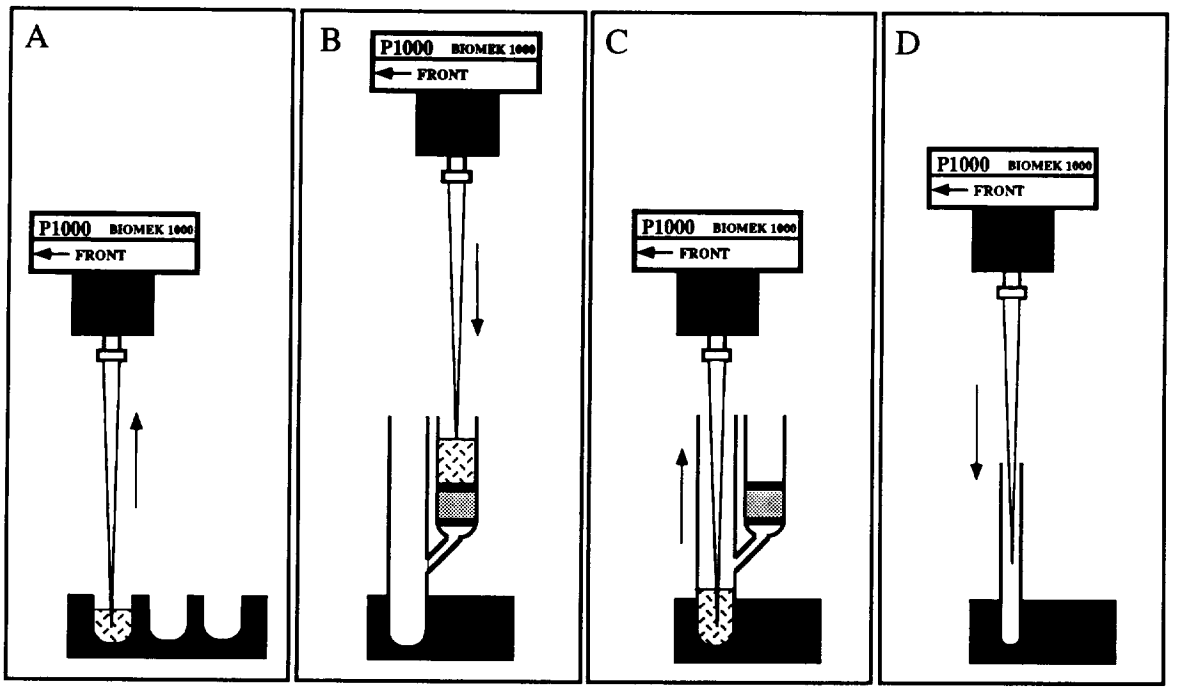

Fig. 2. Major steps automatically performed by the Biomek-1000. The robot pipet tool aspirates the labeled mixture from the thermostatic plate (A), transfers it onto the chromatographic column (B), removes the collected solution from the Y-tube bottom (C) and transfer the material fraction into minitubes (D).

the major drawback of this methodology was the variability of the flow-rate, in relation to the cell number [10,18].

In the present paper, we determined whether automated chromatography through Sephadex G-50 could be reproducibly performed by gravity, using the Y-tube and laboratory workstations, in a routine protocol for the fully automated production of [^{32}P]-labeled molecular probes.

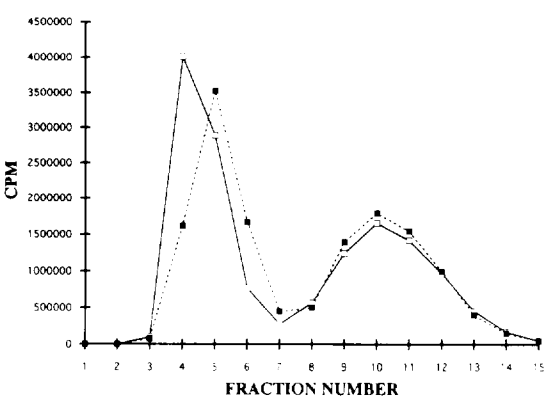

Fig. 3. Comparison of the Sephadex G-50 column elution profiles for the purification of DNA labeled probes, obtained by standard manual procedure (■) and by the fully automated robotic procedure (□).

We demonstrate that the procedure used for DNA labeling by random priming and [^{32}P]-deoxyribonucleotides can be fully automated by using the Biomek-1000 automated laboratory workstation. This procedure involves two steps: the enzyme-directed DNA labeling reaction, performed at constant temperature, and the chromatographic purification of labeled probe by Sephadex G-50 chromatography. We combined the use of vitrified enzymes with the possibility of directly separating the enzymatic products by automatically performed chromatography using the Y-tube. The results obtained clearly show that automatically labeled probe can be efficiently purified by automated Sephadex G-50 chromatography, without any major changes in the hybridization properties.

We believe that these results are of some

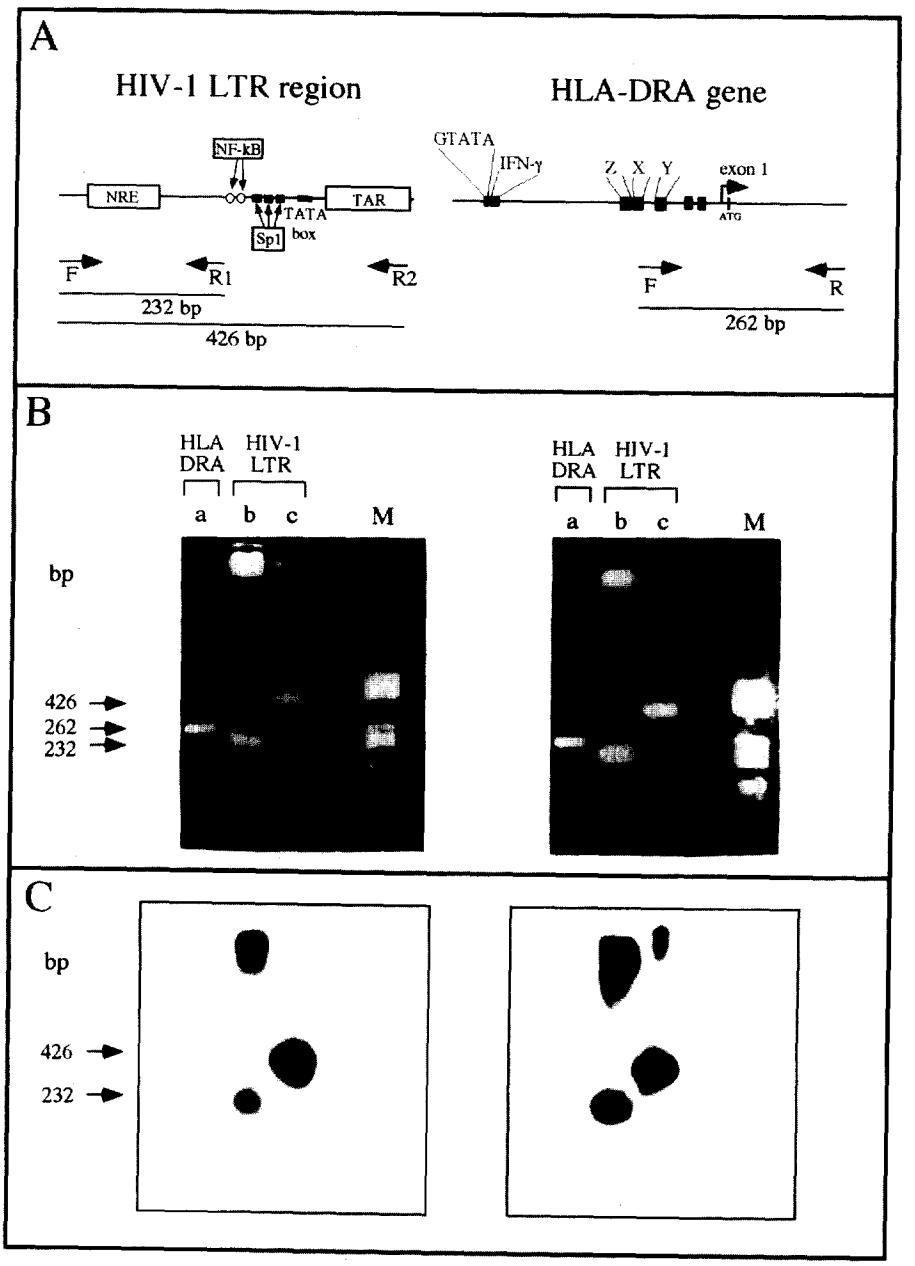

Fig. 4. (A) Location of the primers used for the PCR mediated amplification of the HIV-1 LTR region and HLA-DRA gene. The size of the expected PCR products is also indicated. (B) Agarose-gel electrophoresis of PCR products. PCRs were prepared by using the genomic DNA from the cell line H938 as template and the primers (a) DRA/F-DRA/R for the HLA-DRA gene, (b) LTR/F-LTR/R1 and (c) LTR/F-LTR/R2 for the HIV-1 LTR region. M = molecular mass marker V (Boehringer Biochemica, Mannheim, Germany). (C) Southern blotting hybridization of the gels shown in panel B to the [^{32}P]-labelled HIV-1 LTR probe obtained by manual procedure (left panel) or by fully automated procedure (right panel). Only the specific HIV-1 LTR PCR products hybridize with both the differentially radiolabelled probes.

interest, in view of the variety of biomedical applications of molecular hybridization. It should be noted that, among potentially hazardous materials, radionuclides are commonly used as tracers in most of the molecular biology techniques and in clinical investigations, since radiolabeled molecules allow very sensitive detection. In working with radionuclides, precautions should be taken for handling, including the use of rubber gloves, laboratory coat, plastic-backed adsorbent paper, shieldings [19] and efficient fume hoods [20]. The use of a robotic workstation for the radioisotope handling avoids the exposure of laboratory personnel to radiation. On one hand, good organization of all the reagents needed to complete the radiolabeling reaction and purification helps to minimize radioactive contamination [20]; on the other hand, due to its small dimension, Biomek-1000 and similar robotic workstations can be placed within fume hoods.

Moreover, the automated method could be suitable, with slightly modifications, for other biomedical applications, such as radioiodination procedures of antibodies or antigens for radioimmunoassay (RIA), in which radioactive vapor (^{125}I) is released during the reaction with chloramine-T [20].

In conclusion, we propose this methodology as a safe, fully automated protocol to produce molecular probes commonly used in diagnosis of genetic pathologies and infectious diseases.

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