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Application of capillary DNA chromatography to detect AIDS virus (HIV-1) DNA in blood[a]

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ABSTRACT

The application of capillary DNA chromatography to the detection of the AIDS human immunodeficiency virus, type 1 (HIV-1) proviral DNA in blood is reported for the first time. Combining polymerase chain reaction with fluorescence-labeled DNA probes provides the basis for the amplification and specific detection of the DNA. Samples analyzed for HIV-1 DNA included both infected human blood and cell cultures. The new DNA separation method, capillary DNA chromatography (related to both capillary electrophoresis and capillary hydrodynamic chromatoghraphy), is shown to be a powerful method of analysis for DNA. These preliminary results indicate that an automated approach to screening the blood supply for HIV-1 may become a reality in the future.

INTRODUCTION

Rapid detection and quantification of human immunodeficiency virus, type 1 (HIV-1) proviral DNA in clinical specimens will prove to be increasingly important, both in screening low-risk populations (*e.g.* blood donors) for antibody-negative "silent" carriers of the virus and in the diagnosis and management of high-risk infected persons. Blood banks in the United States screen all potential donors for the presence of the antibodies to HIV-1, but would like to reduce the risk that an infected person who is antibody-negative will slip through antibody screening and self-exclusion criteria. In one study of gay men, 23% were determined by viral culture and conventional polymerase chain reaction (PCR) to be HIV-1-infected although they tested antibody-negative up to eighteen months later [1]. Conventional PCR is still too labor-intensive to be used for each individual donor. In addition to HIV-1, other pathogens such as herpes, human T-cell leukemia, and hepatitis viruses could be added to the PCR-based screening test, if it were faster and more accurate than the conventional gel electrophoresis method.

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The powerful new tool of molecular biology, PCR, can be used to amplify the quantity of a specific piece of DNA if the base sequence is known [2]. Two synthetic oligonucleotides, one encoding the sequence of bases at each end of the target DNA, are used with a special heat-active DNA polymerase from *Thermus aquaticus* (Taq) and a mixture of the single bases in a thermal cycling device. First, the DNA is heated (95°C) to separate the double strand into its single, but complementary single strands (denaturing). Then the sample is cooled (56°C) to anneal the primers to their complementary single strand sequence. At 72°C, the Taq polymerase extends the length of primers along the target DNA sequence. These three steps are called a cycle, and each cycle almost doubles the number of pieces of double-stranded DNA of target sequence. For n cycles, the amplification approaches 2^n.

To ensure that the PCR-amplified DNA has in fact the desired sequence, a different oligonucleotide complementary to the internal target DNA sequences can be used as a probe. This probe is hybridized with the PCR-amplified DNA product to confirm the specific base pair sequence sought. To aid specific detection in conventional PCR, this probe is made radioactive with [^{32}P]ATP.

Gel electrophoresis is routinely used in biochemistry laboratories for the analysis of the PCR-amplified DNA products. Detection of radioactive-labeled DNA probes is accomplished by exposing a sheet of X-ray film to the gel for several hours and then photographically processing the film to make the autoradiogram. Gel electrophoresis suffers from problems: analysis cannot be easily automated; there is poor gel-to-gel reproducibility; and quantitation is imprecise, often only to the closest order of magnitude. The need to work with and dispose of radioactive material adds yet another dimension to the disadvantages of today's gel electrophoresis methods. The need for a modern instrumental method seems clear.

Restriction fragments cover the same size range as PCR products and therefore provide a model with which to develop new analytical methods. Attempts to use high-performance liquid chromatography (HPLC) for double-stranded DNA restriction fragments in this size range [3–5] have not shown the performance of gel electrophoresis in terms of resolution, and hybridization with a labeled probe is precluded due to the lack of multifunction detectors.

Two laboratories [6,7] in 1988 showed the rapid high-resolution separations of DNA restriction fragments by open-tube capillary electrophoresis (CE). This was the first step in demonstrating the practical application of CE to DNA analysis. The present authors have shown a new DNA-specific separation method called capillary DNA chromatography which has resulted in fast separations of DNA restriction fragments with 10 million plates [8]. Both electrophoretic and hydrodynamic separations were utilized. It was anticipated that the analysis of PCR-amplified DNA products should be somewhat less demanding than restriction fragment mapping. Whereas restriction maps require high resolution and excellent reproducibility, PCR produces only one product. Theoretically, if even one

copy of the target DNA is present in the sample, PCR can amplify the quantity of product to the level where it can be detected by the specific probe. In a way, PCR is a go/no go kind of analysis. The very fast hydrodynamic DNA separations might be even more suited to this work than electrophoretic separation.

Beyond the detection in the blood supply of a specific infectious virus, like HIV-1, it would be valuable to be able to determine the viral load in an infected individual. Viral load would be an important factor in the treatment of persons with HIV disease and lead to a better understanding of life history of the disease. The present method of gel electrophoresis of a radioactive sample does not give readily quantifiable results. Even with serial dilutions over several orders of magnitude, the estimation of original viral DNA concentration is problematical.

Much of this uncertainty is due to the use of autoradiography. We hope that with the substitution of fluorescently labeled DNA probes for the traditional ^{32}P-labeled probes and the use of simultaneous UV absorbance and fluorescence detection, the full specificity of gel-based DNA probe and blotting techniques can be preserved. The combined substitution of gel-less capillary DNA chromatography for separation and more precise detection methods should yield a system for rapid, high-throughput, and quantitative analysis of DNA comparable to that which other modern analytical instruments provide.

In this paper, we report the analysis of PCR-amplified DNA and the detection of fluorescent DNA probes using open-tube capillary DNA chromatography. These results, obtained only a year after the first two papers on the separation of DNA restriction fragments by CE, show that capillary DNA chromatography could be of great value in routine DNA analysis.

EXPERIMENTAL

Apparatus

Capillary DNA chromatography was performed in a Model 1200 capillary electrophoresis instrument with Revision 5 software (Microphoretic Systems, Sunnyvale, CA, U.S.A.) The positive-polarity high-voltage power supply furnished with the Model 1200 was used for electrophoresis The hydrodynamic experiments were performed using the M 1200's controlled vacuum system. This system provides vacuum levels from 0.10 bar to a maximum of 0.70 bar with about 3% precision full scale. The instrument incorporates a polychromator and photodiode-array detector with five fixed wavelengths for UV absorption and twelve fixed wavelengths for fluorescence emission. Each photodiode element covers a 14-nm band to maximize sensitivity. The data shown were recorded at 263-nm (for UV absorption) and 534-nm [for fluorescein isothiocyanate (FITC) fluorescence emission] center wavelength. A UG11 broad pass (center wavelength 340 nm) UV filter was used for fluorescence excitation. The Model 1200 acquires data at 10 Hz on up to four channels and stores all raw data first on hard disk and then on tape for archival storage and post run analysis.

Materials

The surface modified (hydrophilic) fused-silica tubing was DB-WAX 60 cm and 120 cm × 100 μm I.D. (J & W Scientific, Folsom, CA, U.S.A.). A 5–8 mm segment of the polyimide coating was carefully burned off the tubing about 2.4 cm from the end and wiped with methanol on tissue paper before installation. Thus cleared, this segment of the capillary provides on-column detection. The running electrolyte was 16 m*M* CTAB (Mixed alkyltrimethyl ammonium bromide), 3.2 *M* urea, 8 m*M* NaH_2PO_4 and 4.8 m*M* NaB_4O_7 at pH 7.2. Oligonucleotides for HIV-1 PCR and detection, SK 38 and SK 39 primers (28 bases each), and SK 19 probe (41 bases probe) [9] were synthesized by the Biomolecular Resource Center (BRC), University of California (San Francisco, CA, U.S.A.). BRC also prepared FITC end-labeled SK 39 and SK 19. The BRC purified all synthetic oligonucleotide products by HPLC Taq polymerase was purchased from Perkin-Elmer Cetus (Norwalk, CT, U.S.A.). A BstN I digest of pBR 322 DNA was purchased from New England Biolabs. (Beverly, MA, U.S.A.) and diluted twenty-fold to 50 μg/ml in 10 m*M* EDTA–0.75 m*M* NaCl buffer. Samples were kept frozen at −20°C until just before use. Buffer solutions were prepared from research-grade components and filtered to remove particulates and to initially degas them.

Procedure for sample preparation and PCR

DNA was extracted [10] from 250 000 peripheral blood mononuclear cells and diluted to a standard volume of 100 μl in the reaction mixture. The reaction mixture was 50 m*M* KCl, 20 m*M* Tris, 2.5 *m* $MgCl_2$ 50 p*M* each of SK 38 and 39 primer, 200 μ*M* each of dNTPs, and 2.5 U of Taq polymerase. To retard evaporation of the reaction mixture, 50 μl of mineral oil were overlaid on the sample prior to PCR.

Samples from two different individuals and two control cell lines were amplified by PCR. The positive HIV-1 control cell line was the promonocyte U1.1 which harbors one copy of the HIV-1 provirus per cell. The negative control was the promonocyte U937, from which U1.1 was derived. A 115 base pair portion of the gag region of HIV-1 was amplified using the SK 38 and SK 39 primers [10]. Thirty-five cycles of PCR amplification were performed on each sample of 100 μl. Each cycle consisted of denaturation at 95°C for 1.5 min, annealing at 56°C for 1.0 min, and extension at 72°C for 2.0 min. After amplification, the samples were extracted with chloroform to remove the mineral oil overlay. For samples to be hybridized, 0.5 p*M* probe SK 19 was added, and hybridization was performed in the dark at 59°C for 15 min.

Procedure for analysis

Samples were gently agitated and placed in the instrument to be ready for automatic injection. The Model 1200 followed a time program of first purging the capillary with the running electrolyte for 3 min, then injecting for 7 s using 0.700

bar of vacuum. For electrophoresis experiments, a positive potential of 10 kV was applied to the sample end of the capillary for 15 min followed by 2 min of vacuum purging of the capillary through the detector. The vacuum step at the end was a control to detect any sample components left in the capillary after the 15-min electrophoresis. Excess dNTPs, primers, and probe should elute together during this vacuum step. Current and optical data were acquired for 17 min.

Hydrodynamic experiments were performed using the same injection conditions and the same buffers as for electrophoresis, but with vacuum used to pull the sample through the capillary. To increase the resolution and accuracy of elution time, a 120-cm capillary was used with 0.10 bar of vacuum for 10 min.

Each PCR-amplified DNA sample was also analyzed by conventional gel electrophoresis using traditional ^{32}P-labeled probes and autoradiography to visualize the sample. Standard DNA restriction standards were run with the double-stranded DNA, and ethidium bromide was used to visualize the separation.

RESULTS AND DISCUSSION

In previous work with restriction fragments [8], we found a set of conditions for the electrophoretic analysis of the expected PCR-amplified DNA of the HIV-1 provirus. A 115 base pair DNA product should elute around 10 min (Fig. 5, ref. 8). In Fig. 1B, the FITC-labeled hybrid elutes at 9.7 min with a clean sharp peak,

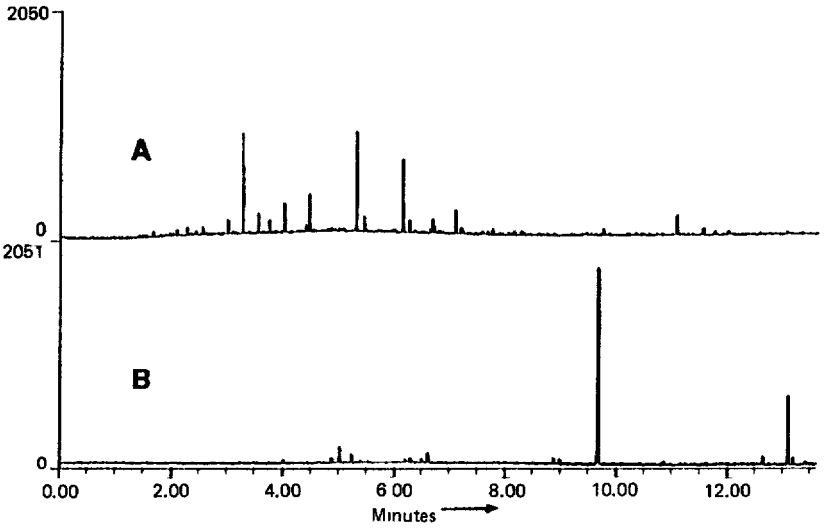

Fig 1 Electrophoretic separation of FITC probe hybridized, PCR-amplified blood samples (A) Sample 18518, HIV-1-negative, (B) individual 1528-3, HIV-1-positive Conditions buffer, 16 m*M* CTAB, 3.2 m*M* urea, 8 m*M* NaH_2PO_4 and 4 8 m*M* NaB_4O_7 at pH 7 2, surface-modified fused-silica capillary tubing, 570 mm × 0.100 mm I.D , applied voltage, +10 kV, 35 μA, detection, 534 nm fluorescence emission, 2050 counts full scale

showing that this individual (1528-3) is positive for HIV-1. The nature of the second FITC-labeled peak at 13.1 min is unknown, but a second band is also present on the autoradiogram when 1528-3 is run on gel electrophoresis with a [^{32}P]probe (data not shown). A second sample, also hybridized with FITC-labeled probe, is shown in Fig. 1A. In this case, in the time window around 10 min there is no significant peak. Therefore this control individual (18518) is negative for HIV-1 provirus. Again, there are artifactual FITC-labeled peaks between 3 and 7 min, but they can be ignored because only the window around 10 min would show the expected 115 base pair product.

In attempt to shorten the PCR sample preparation, we had investigated the use of a FITC-labeled primer, SK 39, and the elimination of the extra step of hybridization with a probe. The results, shown in Fig. 2B, reveal that the positive cell line U1.1 shows a positive FITC product at 10.7 min. The "forest" of other FITC peaks around the main product may indicate imperfect amplification steps with either incorrect sequences and/or base deletions/additions. This is known from our own work with gel electrophoresis and is the reason that probes are used: they provide the extra degree of specificity needed for high confidence in the result. Hybridization with a labeled probe improves the signal-to-noise ratio and decreases the minimum detectable quantity.

The sample TB1-3 shown in Fig. 2A is from an individual shown to be positive for HIV-1 on antibody tests and HIV-1 cell culture, but not consistently positive by replicate runs of conventional PCR using gels and a ^{32}P-labeled probe. One explanation for the mixed results could be that the viral load for this individual

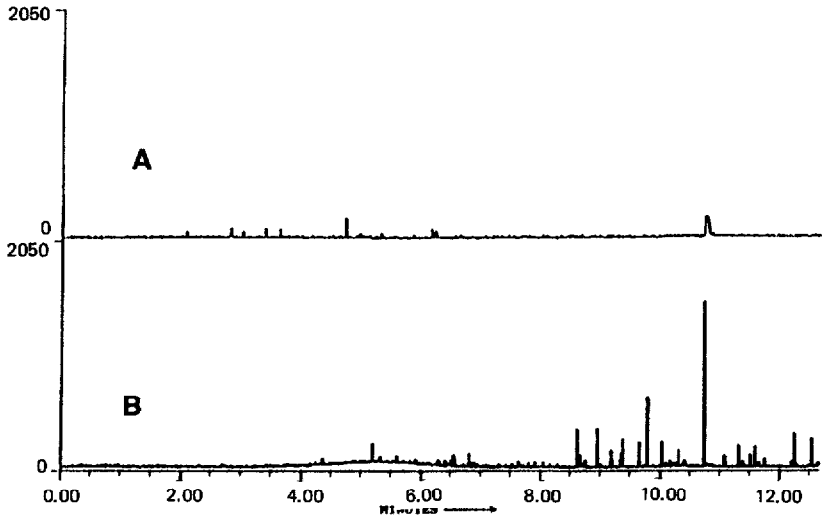

Fig 2 Electrophoretic separation of FITC-labeled primer, PCR-amplified samples. (A) Individual TB1-3, a false negative for HIV-1 by standard PCR with ^{32}P-labeled probe, (B) HIV-1 positive U1 1 cell line Conditions same as Fig 1

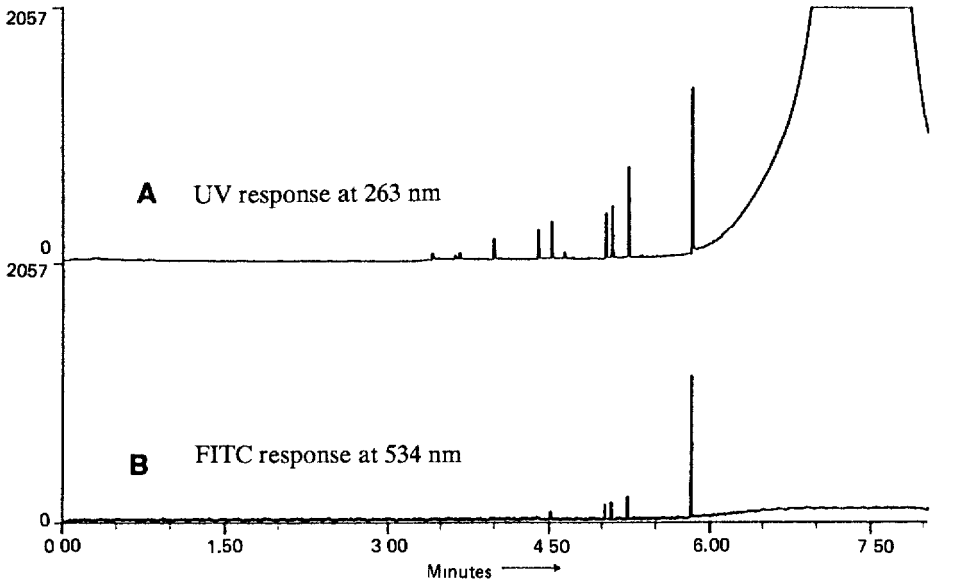

Fig. 3 Hydrodynamic separation of FITC-labeled probe hybridized with PCR HIV-1 product from cell line U1 1 (A) UV response at 263 nm 0 313 o d f s , (B) FITC response at 534 nm, 2^7 scale.

may vary with time. At the time this sample was drawn, the viral load may have been low, hence the small peak at 10.7 min in Fig. 2A. Inspection of this peak shows it to be broader than the strong positive peak in Fig. 1B, which may also be related to the intermittent results by conventional PCR.

The elution time for the FITC-labeled primer PCR product in Fig. 2B is 10.7 min but the elution time for hybridized FITC labeled probe in Fig. 1B is only 9.7 min. This is not due to irreproducibility in the method. Rather, the two products are different. The PCR product in Fig. 2B is 115 base pairs of double-stranded DNA, but the probe-hybridized product in Fig. 1B is only 41 base pairs of double-stranded DNA with the remaining 74 bases left unpaired. There is a difference in mass and charge for these two DNA products.

For the final set of experiments, hydrodynamic separation was used, and the length of the capillary tube was changed to 1.2 m. The set of chromatograms in Figs. 3–5 are shown at the same scale with both UV at 263 nm and fluorescence at 534 nm. In each sample, the excess dNTP's, primers, and probe elute together to produce the large off scale peak in the UV trace. Just below, in the fluorescence trace, a small broad peak can be seen corresponding to the excess FITC-labeled probe. Based upon our previous work with restriction fragments, only double-stranded DNA greater than about 13 base pairs produce the sharp peaks indicative of capillary DNA chromatography. As in size exclusion chromatography, all molecules below a certain size elute together. Figs. 3 and 4 are replicate runs of

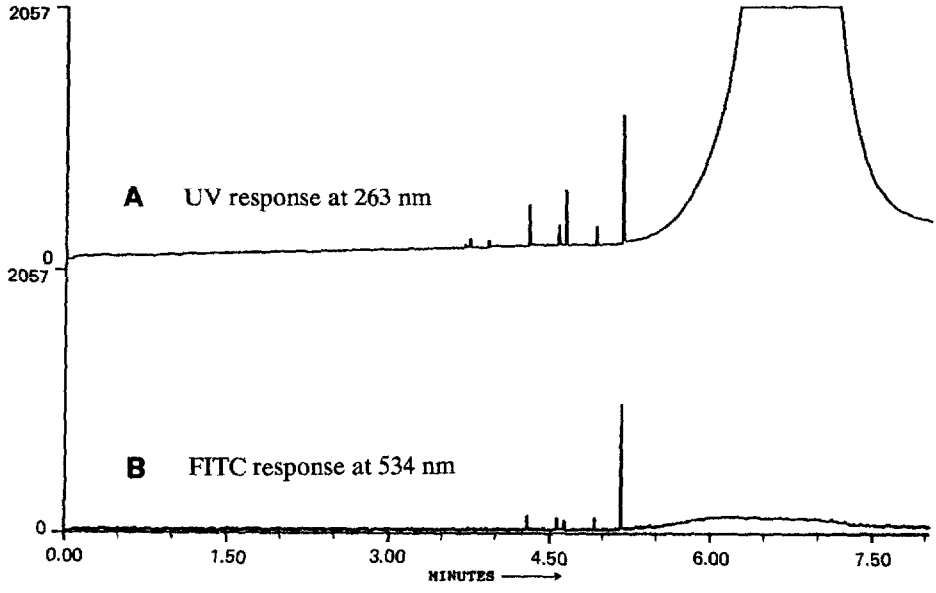

Fig 4 Hydrodynamic separation of FITC-labeled probe hybridized with PCR HIV-1 product from cell line U1.1 Duplicate run of Fig. 3.

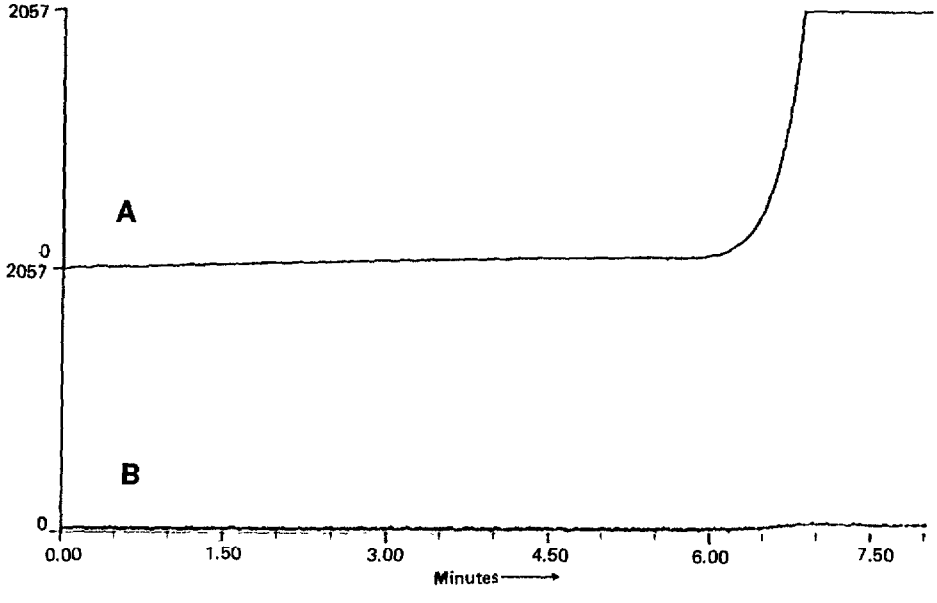

Fig. 5 Hydrodynamic, blank run of PCR reaction mixture (without genomic DNA) after PCR and hybridization For conditions, see Fig 3 (A) UV response, (B) FTIC response at 534 nm

HIV-1 positive cell line U1.1 and show a clear positive signal at 4.8 min in Fig. 3B and 5.2 min in Fig. 4B. When the relative migration times (T_r) are calculated using the solvent peak from the UV traces, both samples yield the same value of 0.778.

The power of a multi-wavelength detector is that more information is available than just the one dimension of separation with time. We believe, because of the work with restriction fragments, that the peaks are separating by size, and we know that every peak in the fluorescence channel must be hybridized DNA. The "blank" run (all steps of PCR amplification and hybridization, except no genomic DNA added) of the reaction mixture containing the FITC-labeled SK 19 probe and the SK 38 and SK 39 primers produces no peaks in either the UV or the fluorescence channel at the expected time, $T_r = 0.778$ (Fig. 5).

When the normalized FITC peak heights are plotted against the ratio of fluorescence to UV (F/A) peak heights, the results shown in Fig. 6 are obtained. The open and closed circles from the data in Figs. 3 and 4 show that the hybridized product stands apart from all other fluorescent peaks. Both the relative intensity of the "other" peaks and the ratios of F/A are smaller than the "correct" hybridized product.

For a given batch of FITC-labeled probe, under constant analytical conditions, there is a fixed fluorescence intensity per mole of probe, and the size of synthetic oligonucleotide probe is fixed. The intensity of the UV signal, however, is directly proportional to the mass of total DNA represented by one peak and therefore indicative of the size of the molecule. It follows, then, that under constant analytical conditions the ratio F/A must be a constant for the "correct" FITC-labeled hybrid PCR product in different samples. Figs. 3 and 4 establish that the ratio F/A in this set of experiments must be about 0.85–1.0. The very small artifact "other" peaks in Figs 3 and 4 would indicate that there is some non-specific hybridization with DNA much larger than the target 115 base sequence. We

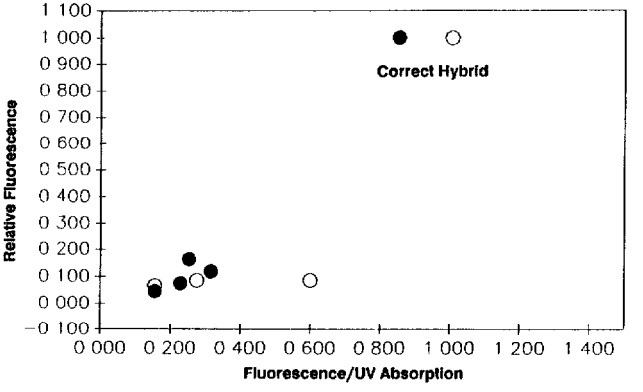

Fig 6 Plots of the ratio of fluorescence to UV absorption on the x-axis against relative fluorescence of FITC peaks Open circles are Fig 3 data, filled circles are Fig 4 data

believe that the artifact peaks are larger than the target for two reasons: (1) they elute before the target and (2) the ratio of F/A is less than the target.

As the mechanism of capillary DNA chromatography is not understood, sample mass overloading may also be responsible for the non-specifically labeled peaks. However, it does serve to illustrate the usefulness of wavelength ratios for confirmation of results. Since we hope to be able to co-amplify the DNA of several pathogens at the same time in the same sample, a complex pattern of fluorescently labeled peaks will not be unexpected in the future, and their discrimination will be required.

These are only preliminary results. Efforts continue in our laboratory to find the best way to solve the challenges posed in the development of a method for the rapid, accurate detection of HIV-1 and other virus in blood. We are hopeful that a fully automated capillary electrophoresis instrument will provide the means once the DNA chemistry under these conditions is better understood and appropriate controls and interpretations are established.

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