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Direct quantification of AD-36 adenovirus DNA by capillary electrophoresis with laser-induced fluorescence

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Abstract

An adenovirus, AD-36, has been linked to human adiposity and a sensitive and reliable quantitative method is required to assess AD-36 viral loads. This report describes direct detection of AD-36 viral DNA, which is the first method to quantitate DNA without amplification. Total genomic DNA is hybridized with an AD-36 specific fluorescently labeled probe and analyzed by capillary electrophoresis with laser-induced fluorescence. The minimum detectable quantity is 10.3 ng/ml, corresponding to 282 copies of AD-36 with a precision of 1–6%. These results indicate that direct detection with capillary electrophoresis with laser-induced fluorescence (CE–LIF) is a reliable and sensitive method for quantifying AD-36 viral DNA.

Keywords: Gene quantification; AD-36 Adenovirus; DNA

1. Introduction

Obesity is rampant in the United States and may soon become a health problem of epidemic proportion in the industrialized world [1]. In the US alone, 54% of adults are overweight and 22% are obese [2]. Although a combination of genetic and environmental factors is thought to cause obesity [3], an emerging theory suggests a role for viruses. Obesity in mice was associated with viral infection as early as 1982 [4] and Dhurandhar and colleagues have recently linked the adenovirus, AD-36, to human adiposity [5]. Additionally, in an animal model these investigators demonstrated that AD-36 was transmitted by blood transfusion, indicating that AD-36 is present and measurable in blood and hinting that adiposity in humans may be transmissible by blood transfusion [6].

Analysis of AD-36 requires a reliable, sensitive and quantitative measure of viral loads in both blood and tissue. The polymerase chain reaction (PCR) is one method to measure viral loads. However, because PCR relies on amplification, limitations in quantitation are inherent [7]. We have previously described a direct detection methodology for HIV, an RNA virus [8]. Direct detection eliminates the amplification step by relying on free solution hybridization of target nucleic acid with specific probes, followed by analysis with capillary electrophoresis with laser-induced fluorescence (CE–LIF). CE–LIF can detect molecules in the attomole range [9] and provides a nonradioactive, fast, reproducible, and

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sensitive method for the analysis of nucleotide fragments [10].

This report describes a direct detection method to quantify AD-36 DNA, and is the first to describe direct detection of DNA with analysis by CE–LIF. First, genomic DNA is obtained by standard methods, and digested with restriction enzymes to generate smaller fragments of DNA. Next, DNA is hybridized with a sequence specific, fluorescently labeled probe, generating a complex of probe and target DNA complex. Finally, the complex is analyzed by CE–LIF. The newly developed procedure allows separation of analyte constituents by molecular size and measurement of fluorescence responses from both the labeled probe and the thiazole orange present in the buffer, which intercalates into the nucleotide complex. Direct detection of DNA by this methodology provides comparable sensitivity with an increased reproducibility over PCR assays.

2. Experimental

All reagents were molecular biology grade and solutions (except buffers used for CE) were autoclaved prior to use. Glassware and disposable supplies were autoclaved prior to use.

2.1. Sample collection and DNA extraction

DNA was obtained from A549 cells (negative control) and A549 cells infected with AD-36 (positive control). A549 cells were obtained from ATCC. DNA was extracted from plasma samples using the Qiagen QIAmp blood or tissue isolation system (Qiagen, Valencia, CA, USA) as recommended by the manufacturer [11]. DNA was resuspended in DEPC-treated water (Biotecx, Houston, TX, USA) and quantitated spectrophotometrically [12]. Cells where counted after trypan blue staining by direct visualization with microscopy (Nikon TMS-F, Melville, NY, USA).

2.2. Probe synthesis

To ensure specificity, a unique gene sequence was probed. Uniqueness was verified by a GenBank search. 5′-Fluorescein phosphoramidite (probe sequence=5′-AGT TGA AAC AGC AAG AGA CTC AAA G-3′, λ=488 nm) labeled and unlabeled DNA probes were synthesized by IDT Laboratories (Coralville, IA, USA).

2.3. Hybridization

Genomic DNA was adjusted to a concentration of 0.5 mg/19.7 μl and digested with six units of MBO1 (1 U is defined as the quantity of enzyme required to digest 1 μg of DNA in 60 min, Promega, Madison, WI, USA) for 2 h at 37°C in the buffer provided by the manufacturer to generate smaller DNA fragments. DNA digests were stored at −20°C until hybridized. Digested DNA (0.44 μg) was hybridized with the DNA probe (1.25 ng) in a buffer volume of 30 ml containing 10 m*M* Tris–HCl (pH 7.2), 1 m*M* EDTA (pH 8.0), 50 m*M* NaCl, and 1 m*M* cetyltrimethylammonium bromide (CTAB) (ACROS, Pittsburgh. PA, USA) [13]. The mixture was heated at 95°C for 5 min and then incubated at 53°C for 6 h. CTAB increases intermolecular crowding and decreases hybridization time [13].

Following incubation, S1 nuclease (2.25 U, 1 unit increases the absorbance of high molecular weight DNA at a rate of 0.001 A_{260}units/ml/min of reaction mixture at 25°C) (Promega) and 0.3 *M* $MgCl_2$ (Promega) were added and samples digested for 20 min at 37°C to destroy single stranded nucleic acids. The reaction was stopped by the addition of 0.4 *M* EDTA and samples stored at 4°C until analysis.

2.4. CE–LIF analysis

Separations were performed on a P/ACE 2050 CE system (Beckman Coulter, Fullerton, CA, USA) at constant temperature (20°C). Detection of hybrids was achieved using laser-induced fluorescence in the reversed-polarity mode (e.g., with the anode at the detector side) at wavelengths 488 nm for excitation and 520 nm for emission. A new sieving polymer was kindly provided by Beckman Coulter. The polymer solution consisted of 25 m*M* MOPS-TRIS pH 7.55 containing 0.5% PEO (mol.wt. 4 million) and 0.4% PEO (mol. wt. 0.9 million). The cathode buffer was prepared by adding 4 μl of thiazole orange intercalator per 10 ml of gel. The capillary was rinsed with the polymer solution containing

cathode buffer for three min prior to each injection. Samples were hydrodynamically injected (10 s) at 0.34 p.s.i. into a 27 cm×75 μm I.D. capillary (effective length of the capillary 15 cm) pretreated with the polymer solution. A water plug for analyte preconcentration (10 s) was injected prior to each sample injection. Separations were performed under constant voltage at 5.4 kV for 15 min. The capillary was calibrated and a standard curve was generated with fluorescently labeled probe and a mixture of appropriately labeled DNA molecular markers (Promega). The markers ranged in size from 36 to 2416 bp. Post-run analysis of data was performed using the System Gold chromatography data system (Beckman).

2.5. Comparison of molecular weight to copy number

One copy of AD-36 DNA consists of approximately 33 068 base pairs (bp). One picogram (pg) of DNA contains 9×10^8 bp, making one picogram equal to approximately 27 216 copies and one attogram approximately equal to 0.027 copies of AD-36 DNA.

2.6. Determination of the optimum injection volume

Based on literature values and our own previous work, the optimum injection volume is 7.1 nl when a sample is injected onto a 100 μm I.D. capillary at 0.34 p.s.i. for 10 s [8,14]. For a 75-μm I.D. capillary, we calculated an injection volume of 5.14 nl for 10 s at 0.34 p.s.i. This injection volume was verified for our system, using the 75-μm I.D. capillary, by measuring the weight loss after injection. An aliquot (40 μl) of hybridization sample was placed in four separate microcuvettes and weighed on a Ohaus GA200D precision balance. Samples 1 and 2 were designated as the sample vials and samples 3 and 4 were used as controls for evaporation. After weighing, the microcuvettes containing the sample were transferred to the auto-sampler tray and injected hydrodynamically at 0.34 p.s.i. for 1980 s (99 s×20 injections, 99 s maximum injection time). The microcuvettes were then re-weighed with a mean decrease in weight after injection of 1050 μg ($n=3$). Since the hybridization solution was very dilute, it was assumed to have the specific gravity of water (1.00 g/ml), corresponding to a mean volume of 1050 nl per 1980-s injection or of 5.3±0.548 nl per 10-s injection ($n=3$).

3. Results and discussion

3.1. Non-specific analysis of AD-36 by total DNA content and restriction digest

We anticipated that AD-36 infected A549 cells would contain more DNA than uninfected A549 cells, indicating the presence of AD-36. To determine if the DNA concentration varied between AD-36 infected and noninfected cells, cells were counted and 2×10^6 of them were aliquoted into fractions for extraction. DNA was isolated, resuspended in double distilled H20 water and quantitated spectrophotometrically.

The A549 cells infected with AD-36 contained a mean concentration of DNA of 1.67 μg/ml ($n=5$). In comparison, the uninfected A549 cells had 1.94 μg/ml ($n=5$) and while there was an absolute difference, there was no significant difference in DNA concentration ($P=0.38$). These data indicate that the AD-36 viral DNA comprises a small fraction of the total cellular DNA, and that comparison of DNA concentrations between infected and noninfected cells is not sensitive enough to identify the presence of AD-36 DNA.

Because the AD-36 DNA sequence differs from the genomic A549 DNA sequence, we expected that a restriction digest would produce a different chromatographic fingerprint in AD-36 infected A549 cells when compared to the uninfected A549 cells and thus allows to distinguish between AD-36 infected and uninfected cells on the basis of the digest with restriction enzymes.

DNA from AD-36 infected A549 cells and uninfected A549 cells was isolated and digested with MBOI and analyzed by CE–LIF. Thiazole orange present in the CE buffer system intercalates double stranded DNA (dsDNA), and thus allows detection of any double stranded DNA, including both genomic A549 and viral AD-36 DNA. No differences in the chromatographic patterns obtained from either

Table 1
Inter and intra day validation of labeled, unlabeled and self-bound probe

Intra-day precision (day 1)	AD36: labeled probe complex	AD36: unlabeled probe complex	Self-bound probe (labeled)
Migration time			
Mean	6.93	6.91	6.61
Standard deviation	0.08	0.11	0.11
C.V.%	1.13	1.61	1.65
Peak area (total)			
Mean	275.79	163.14	152.85
Standard deviation	16.59	20.81	4.05
C.V.%	6.02	12.76	2.65
Inter-day precision (day 1–3)			
Migration time			
Mean	6.68	6.65	6.55
Standard deviation	0.06	0.12	0.13
C.V.%	0.82	1.73	2.05
Peak area (total)			
Mean	204.53	128.22	86.41
Standard deviation	65.12	36.87	58.46
C.V.%	31.84	28.75	67.66

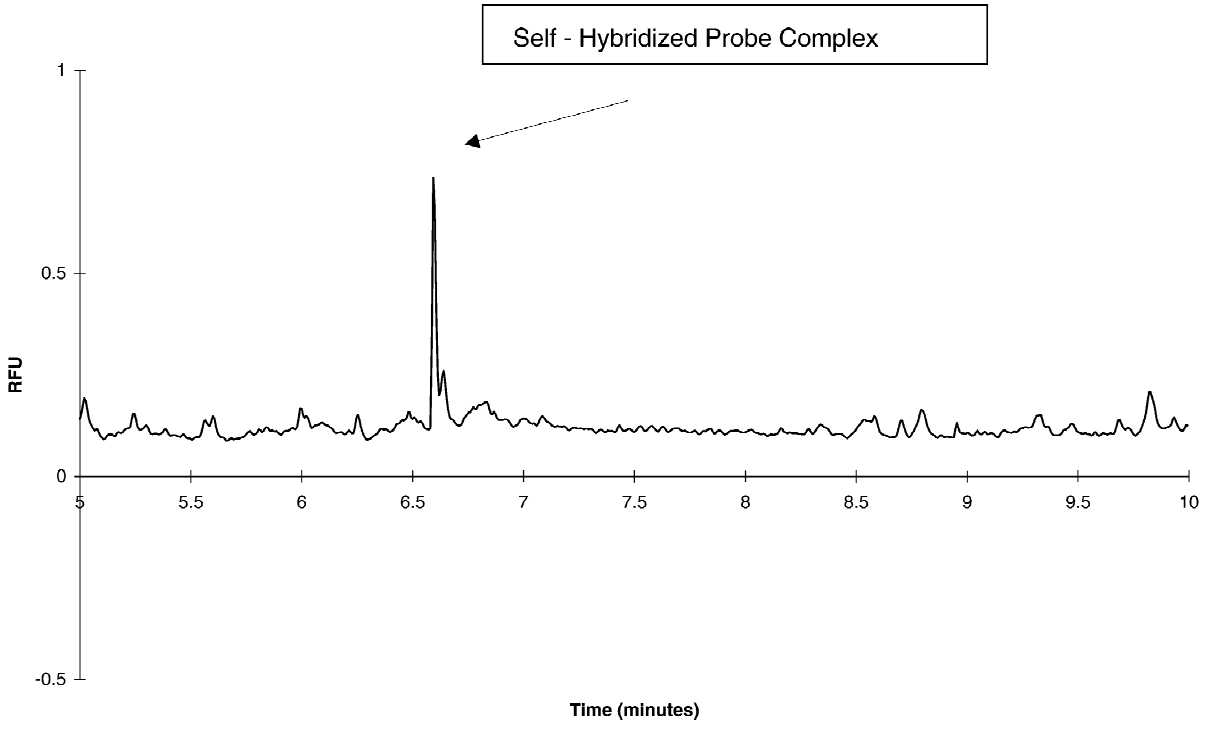

Fig. 1. Electropherogram of AD-36 specific 5′ fluorescently labeled self-hybridized probe. The AD-36-specific labeled probe was hybridized without DNA present and analyzed by CE–LIF. The peak elutes at approximately 6.6 min and represents the self-hybridized probe complex.

the infected or uninfected cells were observed after restriction digest. This likely indicates that AD-36 DNA represented a small fraction of the total cellular DNA and for this reason, is indistinguishable from the total population when analyzed by the restriction digest method alone.

3.2. Specific analysis of AD-36 by labeled and unlabeled probes

To identify AD-36, a unique probe sequence was synthesized and hybridized to target DNA to generate probe–AD-36 nucleotide complexes. Detection of these complexes by CE–LIF depends on measurement of fluorescence from complexes separated by CE–LIF. Thiazole orange present in the buffer intercalates into every one of two DNA bp [14] and fluoresces when bound, representing a single detection system. Labeling at the 5′ position of probes with fluorescein generates an additional signal and in combination with thiazole orange represents a dual detection system.

3.2.1. Analysis of the probe alone

Fluorescently labeled and unlabeled probes unique to the AD-36 virus were synthesized. The probe sequences were identical, the only difference being a 5′ fluorescein. The probes contain the sequence TTGA at the 5′ terminus that may bind to the AACT located at the 3′ terminus of the of the probe,

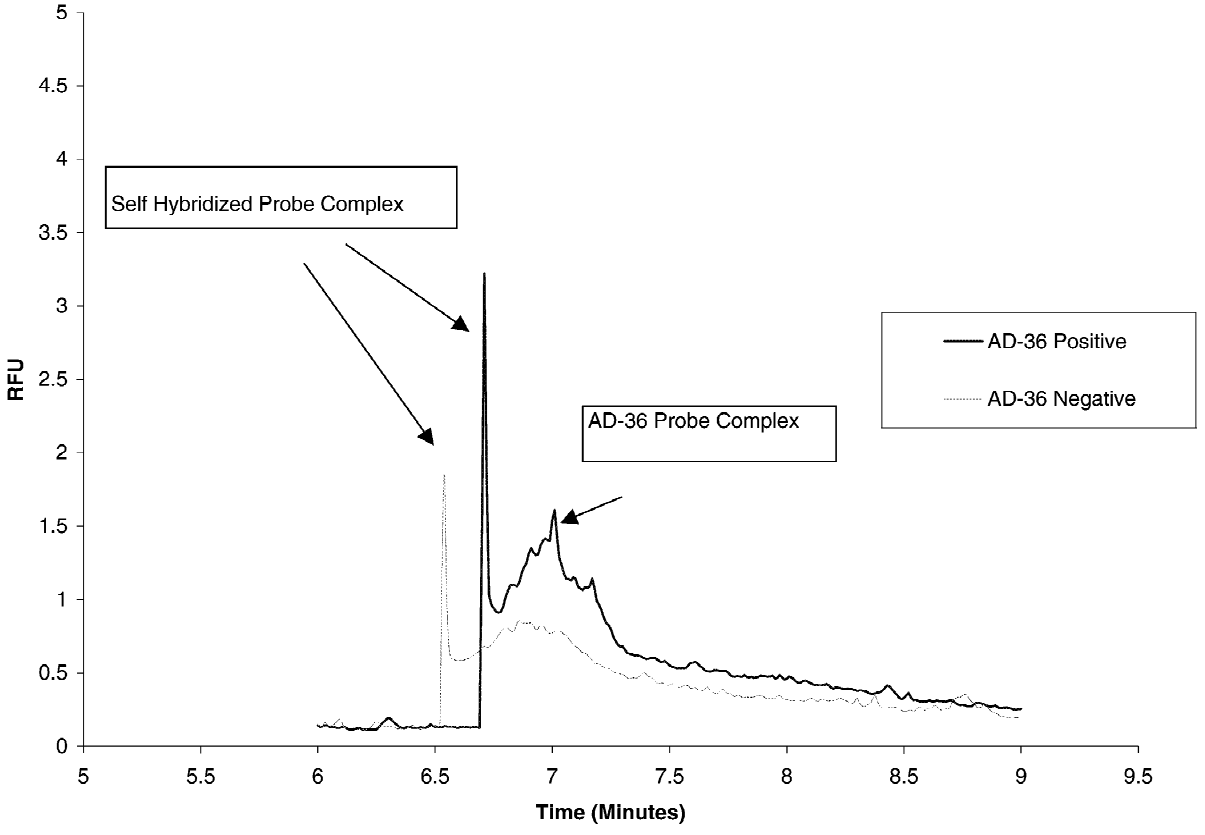

Fig. 2. Electropherograms of AD-36 infected and noninfected A549 cells hybridized with AD-36 specific unlabeled probe. Total DNA obtained from infected and noninfected cells is hybridized with the AD-36-specific unlabeled probe and analyzed by CE–LIF. The first peak eluting at approximately 6.6 min in the AD-36 negative cells and 6.7 min in the AD-36 positive cells represents the self-hybridized probe complex. A mass effect, where increased nucleic acid binding slightly prolongs migration time, may explain the slightly prolonged migration times in the AD-36 positive cells. The second peak eluting at approximately 7 min is present only in the AD-36 positive cells and represents viral DNA.

forming a double-stranded structure which can be intercalated by thiazole orange.

To determine the migration time of the probe, both unhybridized and hybridized, labeled and unlabeled probes were analyzed. The labeled and unlabeled probes were hybridized as described in Experimental, but without target DNA. The final concentration for analysis of both the hybridized and unhybridized probes was 4 pg/μl.

In our system, all dsDNA sequences can be intercalated by thiazole orange and thus generate a fluorescent signal. When the unhybridized, unlabeled, probe was analyzed no signal response was obtained. Therefore, this experiment showed that without hybridization, the probe does not self bind, no dsDNA is formed, no thiazole orange is intercalated and no fluorescent signal is generated.

Both unhybridized, labeled probe sample and hybridized, unlabeled sample generated a single peak eluting between 4 and 5 min. Migration time and peak areas for these samples differ largely yielding values for both precision of peak areas and migration times of >150%. These samples had a single labelling system (either thiazole orange intercalation or 5′ fluorescein). The large C.V. values can be ascribed to substantial analyte degradation under the analysis or storage conditions described above.

The labeled, hybridized, probe generated one peak, eluting at 6.61 min, and indicates the migration time of the self-hybridized probe. This probe com-

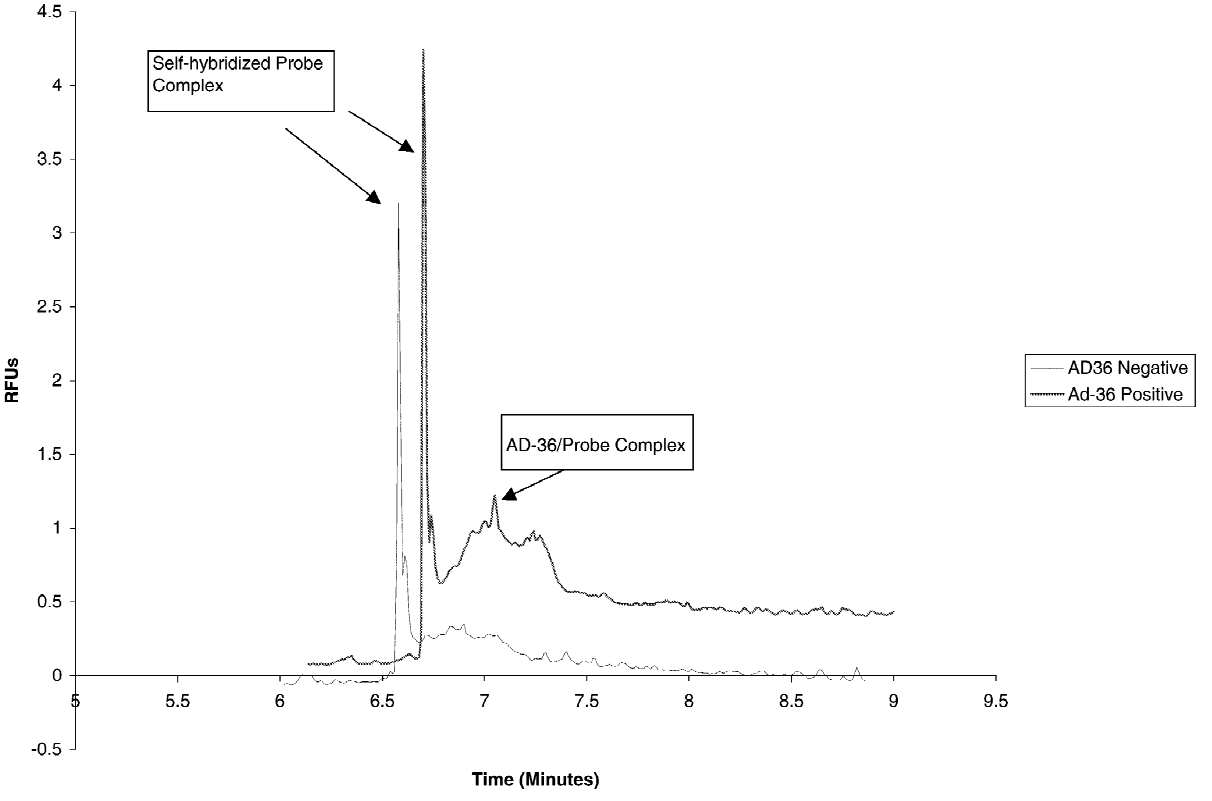

Fig. 3. Electropherograms of AD-36 infected and noninfected A549 cells hybridized with AD-36 specific 5′ fluorescently labeled probe. Total DNA obtained from infected and noninfected cells is hybridized with the AD-36 specific labeled probe and analyzed by CE–LIF. The first peak eluting at approximately 6.6 min in the AD-36 negative cells and 6.7 min in the AD-36 negative cells represents the self-hybridized probe complex. The second peak eluting at approximately 7 min is present only in the AD-36 positive cells and represents viral DNA.

plex was stable with peak area and migrations time coefficients of variation of 2.65 and 1.65, respectively. The lowest detectable quantity of the labeled probe was 10.3 fg/nl, corresponding to 282 copies of AD–36 DNA. (signal-to-noise ratio 3:1) (see Fig. 1 and Table 1).

These data indicate that hybridization as well as dual labeling with fluorescein at the 5′ terminus and intercalation with thiazole orange improves the hybridization complexes stability against degradation and yields markedly better detection sensitivity. The thiazole orange intercalates the nucleotide complexes, and therefore efficiently contribute to inhibit the dissociation of the complex when the high electrical field of CE–LIF is applied. Synergistically, the 5′-terminal fluoroscein moiety also contributes to complex stability, perhaps via protecting the 5′ end against hydrolysis.

3.2.2. Analysis of AD-36 by labeled and unlabeled probe

Both the AD-36 infected and uninfected A549 cells were hybridized with labeled and unlabeled AD-36 probes. A single peak eluting at 6.54 min was identified in the uninfected (AD-36 negative) cells representing the self hybridized probe, whereas two peaks were observed in the AD-36 infected A549 cells, eluting at 6.71 min and 7.01 min, respectively,

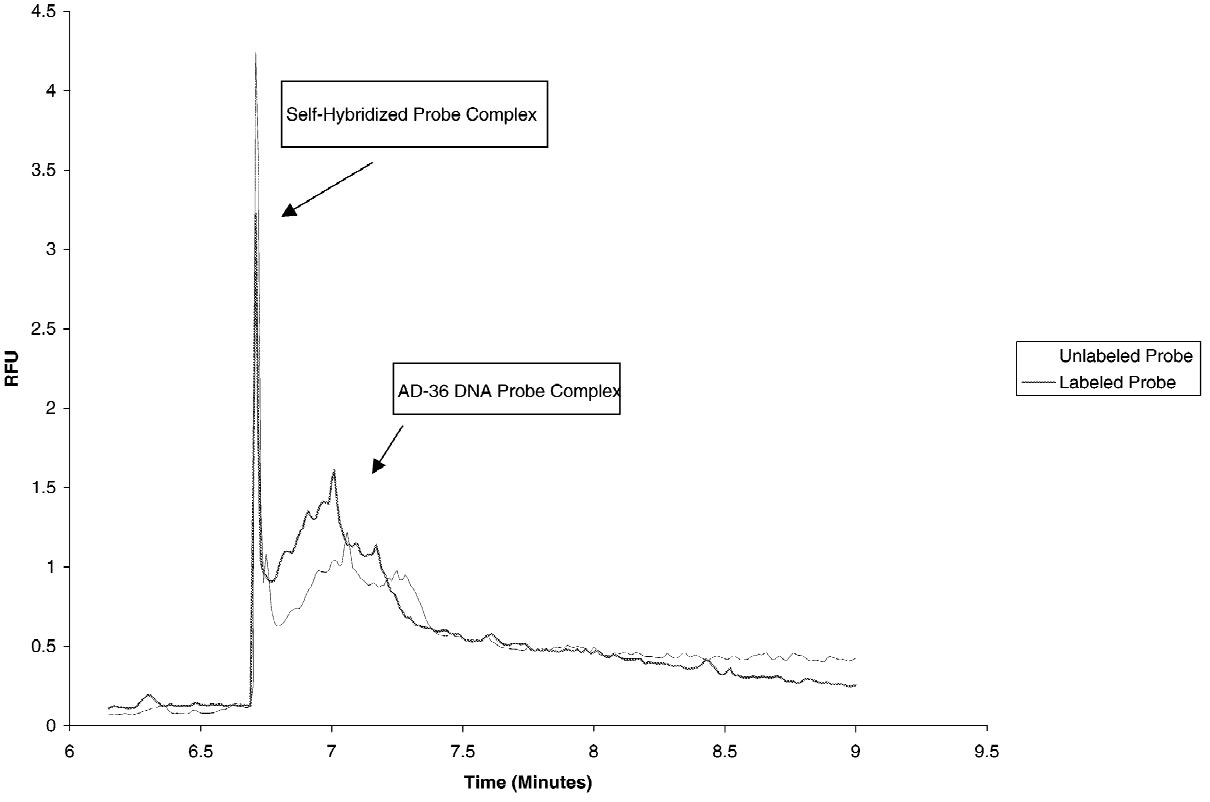

Fig. 4. Comparison of labeled to unlabeled probe in AD-36 positive A549 cells. Total DNA obtained from AD-36 infected cells is hybridized with labeled or unlabeled probe and analyzed by CE–LIF. The first peak elutes in both samples at approximately 6.7 min and is the self-hybridized probe. The second peak eluting for both samples at approximately 7 min is the AD-36 DNA. The unlabeled probe relies on a single detection labeling system, thiazole orange present in the buffer system which intercalates double-stranded DNA and provides the signal. The labeled probe has a dual labeling system, both the thiazole orange and the fluorescent label. The addition of the fluorescent label provides a 59% increase in total peak area.

indicating the presence of both the self-hybridized probe and the AD-36 DNA hybridized with the labelled probe. (Figs. 2 and 3)

Peak areas and migrations time for AD-36 infected A549 cells hybridized with the labeled and unlabeled probe were compared to determine the contribution of the terminal fluorescein over thiazole orange detection alone. (Fig. 4) The terminally labeled fluorescein complexes (fluorescein+thiazole orange detection) generated a mean total peak area of 275.79±16.59 (n=3) compared to that generated by the unlabeled probe (thiazole orange detection only) of 163.14±20.81 (n=3), corresponding to a 59% increase in signal

For the fluorescein labeled probe hybridizations, the intra-day and inter-day migration time precision was 1.13% and 0.82% (n=6), respectively the intra-day peak area precision was 6.0% (n=6) and the inter-day peak area precision was 31.84%. For the unlabeled hybridizations, the intra-day and inter-day migration time precision was 1.61% and 1.73% (n=6), respectively the intra-day peak area precision was 12.8% (n=6) and the inter-day peak area precision was 28.74%. The high values for inter-day precision are presumably attributable to substantial sample degradation under the applied storage conditions.

In addition, the improvement in detection sensitivity was also responsible for an increase in reliability of results, as can be concluded from the decrease in intra-day variability from 12.8 to 6%. The samples were stored at 4°C during the inter-day validation period. Since the inter-day precision was poor at 4°C, attributable to substantial sample degradation, all samples are now run immediately after hybridization.

4. Conclusions

We have developed a direct detection method to quantitate AD-36 viral DNA. The method involves hybridization of total DNA with an AD-36 specific fluorescently labeled probe and analysis by CE–LIF. The dual detection system, consisting of thiazole orange in the buffer system and a 5′-fluorescein labeled probe provides the best sensitivity, with a 59% increase in total peak area attributable to the addition of fluorescein and reliability, improving intra-day peak area precision from 12.76% to 6.01%. This method is readily adaptable to any gene of interest by changing the probe sequence and may be useful for assessing other types of viral loads, including cytomegalovirus, (CMV) and hepatitis C.

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