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# Digital chemiluminescence imaging of DNA sequencing blots using a charge-coupled device camera

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## ABSTRACT

**Digital chemiluminescence imaging with a cryogenically cooled charge-coupled device (CCD) camera is used to visualize DNA sequencing fragments covalently bound to a blotting membrane. The detection is based on DNA hybridization with an alkaline phosphatase(AP) labeled oligodeoxyribonucleotide probe and AP triggered chemiluminescence of the substrate 3-(2′-spiro-adamantane)-4-methoxy-4-(3′′-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD). The detection using a direct AP-oligonucleotide conjugate is compared to the secondary detection of biotinylated oligonucleotides with respect to their sensitivity and nonspecific binding to the nylon membrane by quantitative imaging. Using the direct oligonucleotide-AP conjugate as a hybridization probe, sub-attomol (0.5 pg of 2.7 kb pUC plasmid DNA) quantities of membrane bound DNA are detectable with 30 min CCD exposures. Detection using the biotinylated probe in combination with streptavidin-AP was found to be background limited by nonspecific binding of streptavidin-AP and the oligo(biotin-11-dUTP) label in equal proportions. In contrast, the nonspecific background of AP-labeled oligonucleotide is indistinguishable from that seen with 5′-$^{32}$P-label, in that respect making AP an ideal enzymatic label. The effect of hybridization time, probe concentration, and presence of luminescence enhancers on the detection of plasmid DNA were investigated.**

## INTRODUCTION

Multiplex DNA sequencing, introduced in 1988[1], has the potential of facilitating the processing of large numbers of samples generated in the course of large sequencing projects. The method is characterized by pooling multiple templates prior to performing unlabeled sequencing reactions, submitting the resulting mixtures of DNA sequencing reactions to slab gel electrophoresis, and transfer of the size fractionated fragments onto a blotting membrane. The sequencing information for a specific subset of membrane bound reactions is revealed by hybridizing a labeled oligonucleotide probe specific to the subset of interest to the membrane bound DNA.

While the advantages of multiplex sequencing in reducing the front-end labor of DNA sequencing are widely recognized, the automation of membrane based DNA sequencing presents formidable technical challenges. No fully automated system for this procedure exists today. The major functions of such a system would include direct transfer electrophoresis (DTE)[2–5] for the generation of sequencing blots, automated membrane development and image acquisition, as well as computer based interpretation of these images. Our group is actively pursuing the development of an automated probing chamber for DNA sequencing blots capable of continuous reprobing of sequencing blots without human interaction. A fully automated probing procedure requires the automation of the visualization of the sequencing blots. While images of electrophoretic blots are conventionally acquired on X-ray film, the handling involved in the contact imaging of a blot is not easily automated. Furthermore, X-ray film has to be digitized using a high quality densitometer scanner prior to any computer based evaluation. An alternative to this process is the direct digital imaging of the blots as an integrated stage of the probing cycle, using modern digital imaging sensors instead of film as the recording medium. Independent of the particular implementation of a digital imager into an automated probing procedure, direct digital imaging of electrophoretic blots is generally useful because it allows the direct subsequent computer interpretation of the data[6–9]. This study describes direct digital imaging of DNA sequencing blots in an automatable fashion based on a charge-coupled device (CCD) camera.

The introduction of new enzyme triggered chemiluminescent substrates has significantly improved the nonisotopic visualization of membrane bound DNA[10,11]. In particular, stable 1,2-dioxetanes like the alkaline phosphatase(AP) substrate 3-(2′-spiroadamantane)-4-methoxy-4-(3′′-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD) introduced in 1987 [12,13] have proven useful in many applications. For membrane based DNA sequencing, AMPPD offers the advantage of being significantly more sensitive than colorimetric detection[14], approaching the sensitivity obtained with $^{32}$P-labeled oligonucleotide probes. The exposure time of chemiluminescent blots to X-ray film are significantly reduced compared to the time required for autoradiography. The 'glow'-type chemiluminescence of 1,2-dioxetanes allows multiple exposures of a blot and adjustment

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of the exposure time to variations in sequencing band intensity caused for example by the template dependency of the sequencing chemistry and variations in the blotting and probing efficiency. Because of these advantages, the use of AMPPD[5,15–17] and its chloride-substituted derivative CSPD[18], has been described for membrane based DNA sequencing. Two of these studies[16,17] used DNA-DNA hybridization of probes for detection, allowing multiplex sequencing, while the use of sequencing primers labeled with biotin[5,18] and digoxigenin[15] is not compatible with this strategy. Detection by enzyme triggered hybridization assays has been facilitated and improved by the development of direct oligonucleotide-AP conjugate probes[19–22]. The use of these probes reduces the number of incubations and washing steps in the membrane development procedure, compared to strategies involving oligonucleotide probes labeled with secondary reporter molecules such as biotin or digoxigenin.

A further advantage of chemiluminescence visualization of DNA sequencing blots is the compatibility with modern high sensitivity imaging sensors. Cryogenically cooled, low readout noise CCD cameras are particularly well suited for scientific low light level imaging[23,24], because of their high sensitivity, linear response to incident photons, and wide dynamic range. While CCD's have been used for direct digital chemiluminescence imaging of Southern- and dot-blots [25–28], applications have generally focused on obtaining high sensitivity and quantitative information. Imaging of large DNA sequencing blots as described in this study, however, requires high sensitivity per unit time as well as high image resolution and spacial fidelity to resolve the tightly spaced bands on these blots while maintaining an acceptable sampling speed.

To establish a protocol for CCD chemiluminescence imaging of DTE sequencing blots, we initially investigated the hybridization based detection on slot blots, comparing AP- and biotin-labeled oligonucleotides as hybridization probes, and the reagents causing the background limited detection seen with the biotin label. The effect of hybridization time, the concentration of probe in the hybridization buffer, and the addition of luminescence amplifying additives to the substrate solution on the detection characteristics were studied using the oligonucleotide-AP probe. The sequencing method involves Sanger dideoxynucleotide DNA sequencing[29] of double strand plasmid DNA, generation of sequencing blots by direct transfer electrophoresis, hybridization with oligonucleotide-AP probes and digital chemiluminescence imaging.

## MATERIALS AND METHODS

### Preparation of oligonucleotide probes and slot blots

All hybridization probes are based on the same 5′-AA GGTGTGGGGTAGGATGGTT-3′ (20-mer, $T_m = 2(A+T)+4(G+C)°C = 62°C$) oligonucleotide. A sequence complementary to the probe is found in the identifier region on one of the plasmid vectors in our multiplex DNA sequencing vector set [30]. The oligonucleotide serves to detect this vector in the slot blot experiments as well as DNA sequencing fragments generated with the vector serving as template.

The biotinylated probe is obtained by enzymatic tailing of the synthetic oligonucleotide using terminal deoxynucleotidyl transferase (TdT). In the labeling reaction 10 μg (1.4 nmol) oligonucleotide is mixed with 25 nmol biotin-11-dUTP (Sigma), 60 units TdT (17 U/μl, Pharmacia), and 4 μl 5× $CoCl_2$ tailing buffer (500 mM potassium cacodylate (pH 7.2), 10 mM $CoCl_2$, 1 mM dithiothreitol, obtained from BRL) in 20 μl total volume and incubated at 37°C for 4 h. The extension products are purified from unreacted oligonucleotide and excess biotin-11-dUTP by preparative electrophoresis in 20%T, 5%C polyacrylamide gel, eluted in Maxam & Gilbert elution buffer (0.5 M $NH_4$ acetate, 10 mM Mg acetate, 1 mM EDTA, 0.1% SDS), desalted by ethanol precipitation, and quantitated by its 260 nm absorbance. An aliquot of the reaction mixture is 5′-$^{32}$P-labeled and electrophoresed separately on the preparative gel to determine the length of the oligo(biotin-11-dUTP) tail by autoradiography. On average 3 biotin-11-dUTP residues are attached to the oligonucleotide by this procedure, which is taken into account in quantifying the probe concentration.

Oligonucleotides are 5′-end labeled with [γ-$^{32}$P]ATP (NEN) and T4 polynucleotide kinase (Pharmacia) using a standard protocol[31]. Excess radioactive label is removed with high efficiency from the biotinylated and non-biotinylated probe by a NucTrap pushcolumn (Stratagene, La Jolla, CA).

The 1:1 oligonucleotide-AP conjugate is produced by attaching AP to 5′-amino-oligonucleotide 5′-$NH_2$-$(CH_2)_6$-AAGGTGT-GGGGTAGGATGGTT-3′ via a homobifunctional crosslinker as described[19]. The 5′-amino oligonucleotide is synthesized on a 380A oligonucleotide synthesizer using Aminolink 2 reagent (both Applied Biosystems, Foster City, CA). The purified oligonucleotide-AP conjugate used in the experiments is free of unlabeled oligonucleotide and unreacted AP. The concentration of the conjugate stock solution is 6000 nM. AP-activity as determined by the p-nitrophenyl phosphate (pNPP) assay (6 mM pNPP (Sigma 104) in 10 mM glycine, 1 mM $MgCl_2$ buffer, pH 10.4, 37°C) is 520 U/ml.

Slot blots are prepared by applying a 1:2 dilution series of circular plasmid DNA (2.7 kb plasmid) denatured in 0.2 N NaOH onto the membrane previously equilibrated in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3). Throughout this study, uncharged Biodyne A nylon membrane (Pall Biosupport) with 1.2 μm pore size is used. A Hybri-Slot-Manifold (BRL, Gaitherburg, MD) slot blot apparatus is used to deposit the target DNA on 4×0.5 mm areas on the membrane. The solution in the wells is filtered through the membrane by mild suction. The denatured plasmid-DNA is cross-linked to the membrane using a U.V. dose of 120 mJoule/cm$^2$ in a UV Stratalinker (Stratagene), and dried for 10 min at 60°C before storage.

### Sequencing of plasmid DNA

Enzymatic dideoxynucleotide DNA sequencing reactions are performed using the pUC derived 2.7 kb double-stranded multiplex sequencing vector as template. This vector provides priming and unique recognition sites for oligonucleotide hybridization at both sides of the insert to be sequenced. For each sequencing reaction 5 ng of unlabeled primer (18-mer), 1 μl 10× reaction buffer, and 2.5 μg of CsCl purified, denatured template DNA are combined to a total volume of 10 μl. The 10× reaction buffer is 400 mM Tris pH 7.6, 100 mM $MgCl_2$ and 500 mM NaCl. For annealing, the solutions are heated to 72°C, and slowly cooled to room temperature over a period of 1 h. 1 μl 0.1 M dithiothreitol, 1.5 μl 10× reaction buffer, 1 μl water, and 2 μl of a 1:8 dilution of Sequenase Version 2.0 (13 U/μl, USB) are added to the template-primer solution and mixed. For the termination reactions 2.5 μl of each of the four d/ddNTP mixtures are placed in separate tubes prewarmed to 37°C, and 3.5 μl of

the template-primer mixture containing the enzyme is added to each tube. All d/ddNTP mixtures are 80 μM dNTP. The d/ddCTP mixture is 4 μM ddCTP, the d/ddATP mixture is 4 μM ddATP, the d/ddGTP mixture is 8 μM ddGTP, and the d/ddTTP mixture is 8 μM ddTTP. The termination reactions are carried out at 37°C for 10 min before the enzyme is inactivated at 72°C for 10 min. 4 μl formamide stop solution is added to the samples before heating to 95°C for 5 min, and loading onto the sequencing gel.

The DTE apparatus used for the generation of DNA sequencing blots is comparable in design to those described by Pohl et al.[2–4], and to commercially available direct blotters, and was constructed in house. The sequencing reactions are separated on a 40 cm long, 100-to-200 μm reverse wedge, 4% Long Ranger (AT BioChem Inc., Malvern, PA) slab gel by applying a 70 V/cm electrical field, and directly transferred to a Biodyne A membrane moving at a blotting speed of 4 mm/min when eluting from the gel. Cross-linking and membrane development (hybridization in 6 nM oligonucleotide-AP) are as described for the slot blots.

### Membrane development for detection

The development procedure is an adaptation of published methods for membrane based DNA hybridization assays using oligonucleotide-AP probes [14,16,19] and the Southern blot hybridization procedure given for commercially available probes (SNAP, Molecular Biosystems, San Diego, CA). Blots to be probed with the oligonucleotide-AP conjugate are pre-hybridized in PBS-SB buffer (PBS buffer: 40 mM potassium phosphate, 130 mM NaCl, pH 7.6; PBS-SB buffer: 1% sodium dodecylsulfate (BioRad, enzyme grade), 0.5% bovine serum albumin (Sigma) in PBS buffer) for 15 min at 50°C. Hybridization is carried out by incubating the blots in oligonucleotide-AP in PBS-SB buffer (5 ml/100 $cm^2$) for 15 min at 50°C followed by one wash in PBS-SB buffer for 10 min at 50°C and three washes in PBS buffer for 10 min each. The blots are then equilibrated in DEA buffer (0.1 M diethanolamine, 1 mM $MgCl_2$, 0.02% $NaN_3$, pH 9.6) for 10 min, and subsequently incubated in 0.40 mM AMPPD (Tropix Inc., Bedford MA) in DEA buffer (5 ml/100 $cm^2$) for 10 min in a heat-sealable plastic bag. Excess AMPPD solution is drained off, and the moist membrane is sealed in the bag. The enzymatic reaction is allowed to proceed for 6–8 h prior to recording CCD images.

Blots to be probed with biotinylated oligonucleotide are pre-hybridized in PBS-SB for 15 min at 50°C, and incubated in oligo(biotin-11-dUTP)-oligonucleotide in PBS-SB for 15 min at 50°C, followed by one wash in PBS-SB buffer for 10 min at 50°C, and two washes at room temperature for 10 min each. The blots are incubated in a 1:10000 dilution of streptavidin-AP conjugate (1000 U/ml, Boehringer Mannheim) in PBS-SB in a shaking tray for 15 min, washed once in PBS-SB for 10 min, and 3 times in PBS for 10 min. The subsequent handling, starting with the equilibration in DEA buffer, is the same as described for the AP-labeled probes.

The membranes are washed in shaker trays with excess liquid, while heat-sealable bags are used for hybridization and incubation in AMPPD solution. All developing steps are carried out at room temperature unless otherwise stated.

### Digital chemiluminescence imaging and autoradiography

Chemiluminescent images are acquired by a CH210 CCD camera (Photometrics Ltd., Tucson, AZ), equipped with a 576×384 element TH7882CDA CCD-array (Thomson CSF), operated at −120°C under $N_2$-cooling. The camera is operated via the CC200 controller hardware and software provided by Photometrics. The digital images are stored in the original 2-byte binary data type with a storage requirement of 0.44 Mbyte per frame. Data evaluation is performed using routines written in Pro-Matlab (The Mathworks Inc., South Natick, MA) matrix oriented numerical calculus software. A standard Pentax-A camera 50 mm focal length lens set to f/1.2 is used to image the blots at the shortest lens-to-object distance that can be focussed, about 36 cm.

The optical transfer properties of the camera are dominated by three factors, the light collection efficiency of the lens c.e. (0.087%, as calculated using c.e. = $m^2/16F^2(m+1)^2$ where F and m are the numerical aperture and the magnification resp.), the quantum efficiency of the CCD detector $\Phi_{detector}$ (0.18 photoelectrons per incident photon at 460 nm, as from the specification chart of the TH7882 CCD array) and the lens transmittance $\tau$ (93%, as determined using a 488 nm laser beam). The number of photoelectrons $N_{pe}$ generated on the CCD by an isotropic light source emitting $N_{photons}$ is given by,

$$N_{pe} = \tau \, \Phi_{detector} \, c.e. \, N_{photons}$$

The overall optical transfer efficiency considering these three factors is $0.14*10^{-3}$ ($\tau \, \Phi_{detector}$ c.e.) in our system. About 7000 photons have to be emitted from a point source in the sample plane for every photoelectron generated on the CCD surface. The incident light flux at the CCD surface generated by a particular experiment in this study can be derived from the CCD counts given by using the system gain factor of 16.76 photoelectrons/count.

Digital autoradiograms are recorded after exposing radioactive blots to a storage phosphor screen for 30 min, and subsequently scanning the screen with a PhosphorImager(PI) scanner, (both Molecular Dynamics, Sunnyvale, CA). The method has been shown to generate signal linear to the incident radiation of $^{32}P$ of over five orders of magnitude [32], and thus ideally suits the purpose of quantitative autoradiography. PhosphorImager and CH210 images are evaluated with the same software.

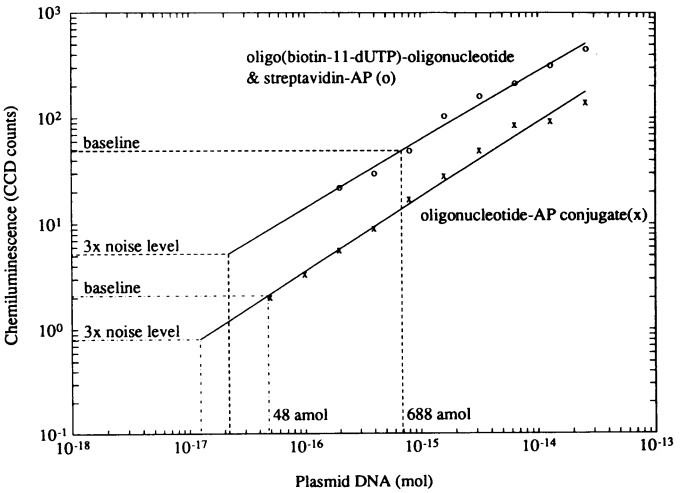



**Figure 1.** Chemiluminescence standard curves obtained with AP- and oligo(biotin-11-dUTP) labeled oligonucleotides. Detection limits are 6.8 pg (AP) and 12 pg (oligo(biotin-11-dUTP)) of 2.7 kb plasmid DNA. Conditions: 1.2 nM probe in hybridization buffer, 30 min CCD exposure).

## RESULTS AND DISCUSSION

### Alkaline phosphatase- versus oligo(biotin-11-dUTP)-labeled oligonucleotide probes for chemiluminescence detection of plasmid DNA

Slot blots with dilution series of cross-linked plasmid DNA were processed with equal amounts of the two probes (1.2 nM in hybridization buffer) according to the procedures given under Methods. Fig. 1 shows the response curves, baseline levels, and noise estimates observed on these membranes.

To determine the chemiluminescence detection characteristics, a one dimensional lane is extracted from the digital image by averaging across the full width of the bands. For all detectable bands on the lane, consisting of the dilution series spots, the chemiluminescence response is calculated as the height of the peaks above baseline. The standard curve then relates the chemiluminescence signal x to the amount of target DNA y $\log_{10}(y)=a_0+a_1\log_{10}(x)$. In addition to the standard curve, the probing procedure is characterized by its baseline response, which is measured relative to a CCD dark exposure of the same integration time. The baseline response is identical to the image background. To further characterize the detection, the baseline noise is determined and used to establish the limit of detection (3× noise level). The noise of the image background, that is the noise associated with a single CCD element, is generally much higher than the baseline noise levels, because of the averaging across the width of the bands.

Evaluating Fig. 1, we see that the detection using the biotinylated probe is limited by background luminescence caused by nonspecific binding to the membrane. The background seen on the blots developed with oligonucleotide-AP direct conjugate corresponds to only 47 amol of plasmid DNA (1 amol = $10^{-18}$ mol, 1.8 pg plasmid DNA), while the background found on the

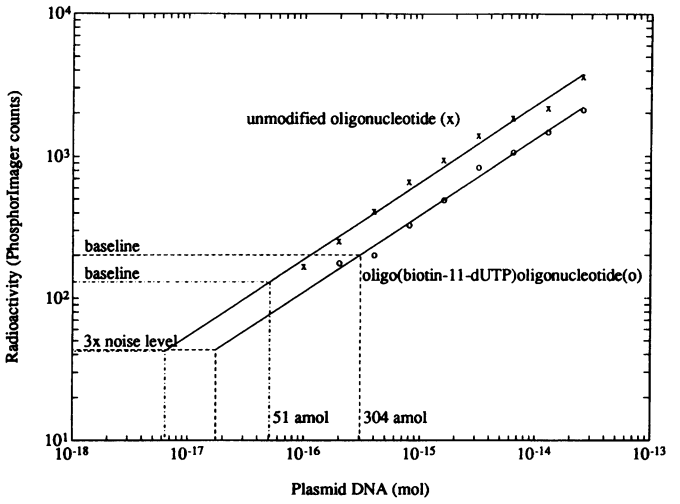



**Figure 2.** Radioactivity standard curves of $^{32}P$ 5'-end labeled biotinylated and non-biotinylated oligonucleotide. Conditions: 1.2 nM probe in hybridization buffer, digital autoradiogram obtained by 30 min exposure to a phosphor screen and PhosphorImager(PI) scanning.

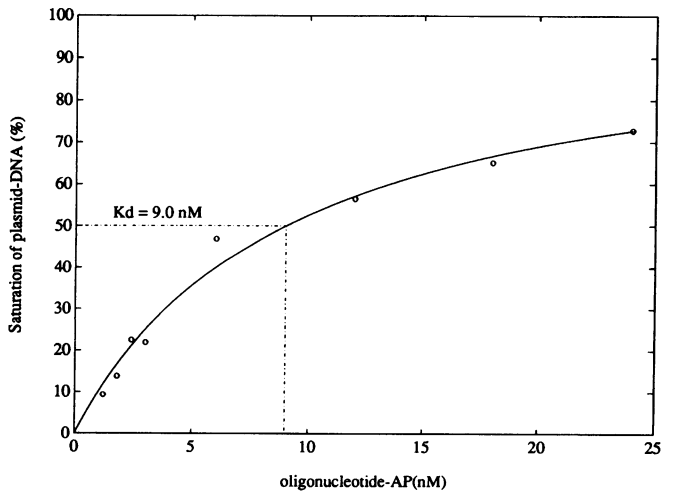



**Figure 3.** Saturation isotherm of oligonucleotide-AP binding to membrane bound plasmid DNA (1 fmol) normalized for maximum binding. The concentration of probe in the hybridization buffer varied from 1.2–24 nM. Conditions: 1.2 nM probe in hybridization buffer, 15 min hybridization, chemiluminescence detected on 30 min CCD exposures.

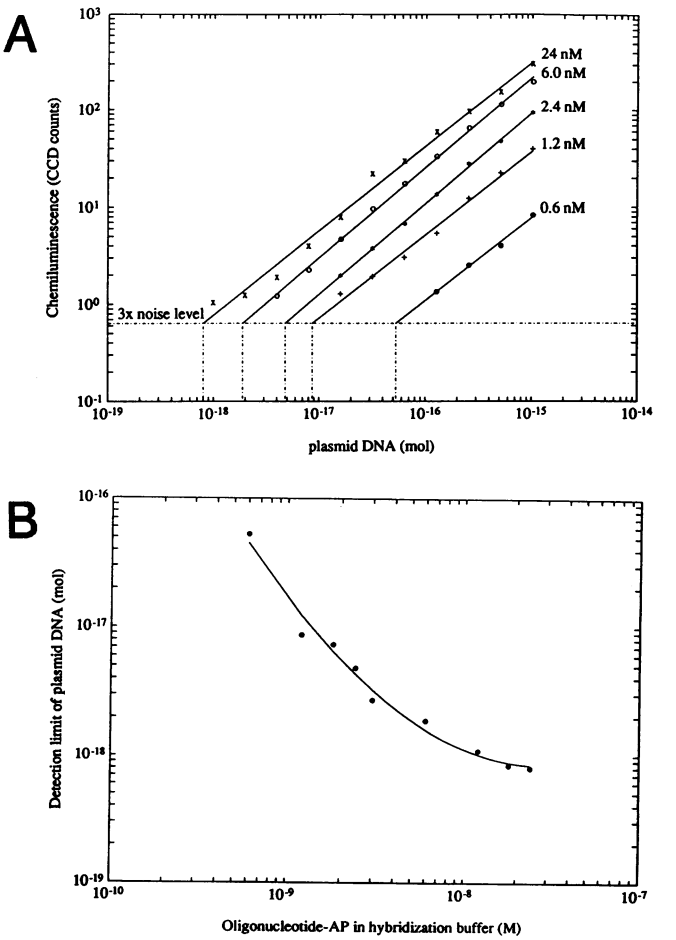



**Figure 4.** Effect of the concentration of oligonucleotide-AP in the hybridization buffer, 15 min hybridization time, 30 min CCD exposure time. **A.** Standard curves obtained with different concentrations of oligonucleotide-AP probe in hybridization buffer. **B.** Detection limit (S/N=3) for plasmid DNA with probe concentration varying from 0.6 nM to 24 nM. Lowest detection limit corresponds to 0.5 pg of the 2.7 kb plasmid.

biotin/streptavidin-AP treated blots corresponds to 688 amol plasmid DNA. Notice that the higher background level results in increased noise which in turn results in a less favorable 21 amol detection limit of the biotin label compared to 12 amol for the oligonucleotide-AP. This is particularly striking considering that the biotin based detection features 3-to-4-fold greater response than the oligonucleotide-AP, reflected in the offset of the two response curves in Fig. 1. The higher light level can be attributed to the multiple biotin residues on the oligonucleotide, the high AP activity of the streptavidin-AP reagent, less inactivation of AP during membrane development because the enzyme is not submitted to the stringent hybridization conditions, and potentially better binding of the probe to the target-DNA due to its smaller size. As is true for shot noise limited luminescence measurements in general, an increase in signal intensity will only trade into better detection limits, if it can be obtained without significant increase in background luminescence.

The limitations to the detection of plasmid DNA with the biotin/streptavidin-AP protocol originate from the high level of background luminescence and can be more clearly understood by identifying the reagent causing the nonspecific binding. To quantitate the contribution of the oligo(biotin-11-dUTP) portion of the probe to the background luminescence, native and biotinylated oligonucleotides are radioactively labeled, and used to probe slot blots which are evaluated by PhosphorImager autoradiography as described under Methods. The same concentration (1.2 nM in hybridization buffer) of probe is used as for the chemiluminescence detection. The result of this experiment is shown in Fig. 2. The response curves of the two probes are slightly offset, presumably due to differences in the efficiency of [$\gamma$-$^{32}$P]ATP labeling and probe binding. Because a difference in the specific activity of the probe will affect signal and background to the same extend, we can nevertheless determine the contribution of oligo(biotin-11-dUTP) to the background from this data by calculating the difference in the amounts of target corresponding to the two background levels. The presence of oligo(biotin-11-dUTP) on the oligonucleotide probe increased the background by what corresponds to 304−51 amol = 253 amol. Notice when comparing the noise levels of the PhosphorImager in Fig. 2 to those seen with the CH210 in Fig. 1, that the PhosphorImager data is detector noise limited and the detection limits therefore independent of the baseline level.

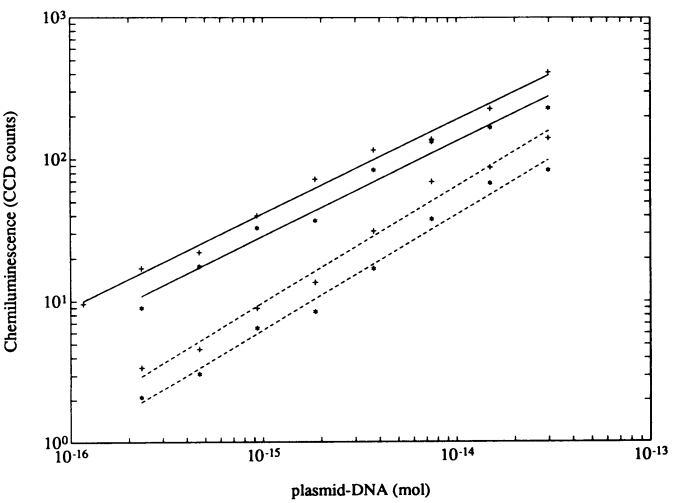



**Figure 5.** Chemiluminescence response using 0.4 mM AMPPD in diethanolamine buffer (—) and in carbonate buffer (--). Non-enhanced luminescence (*) and luminescence in the presence of 'Sapphire' enhancer solution(+). Conditions: 1.2 nM probe in hybridization buffer, 10 min CCD exposures.

Three sources of the high background seen with the biotin/streptavidin-AP protocol can be identified. A significant 36% (250/688) portion of the background is due to nonspecific binding of the biotin residues of the biotinylated probe. The second major contribution can be attributed to the binding of streptavidin-AP conjugate to the membrane, which is not completely prevented even in the presence of 1% SDS in the incubation buffer. The background not attributable to either one of the two sources corresponds to 47 amol and is due to nonspecific binding of the oligonucleotide or AP portion of the probe, and buffer or membrane contaminants. Compared to the two major sources, the residual background only represents 7% (47/688) of the total background in the biotin/streptavidin-AP protocol.

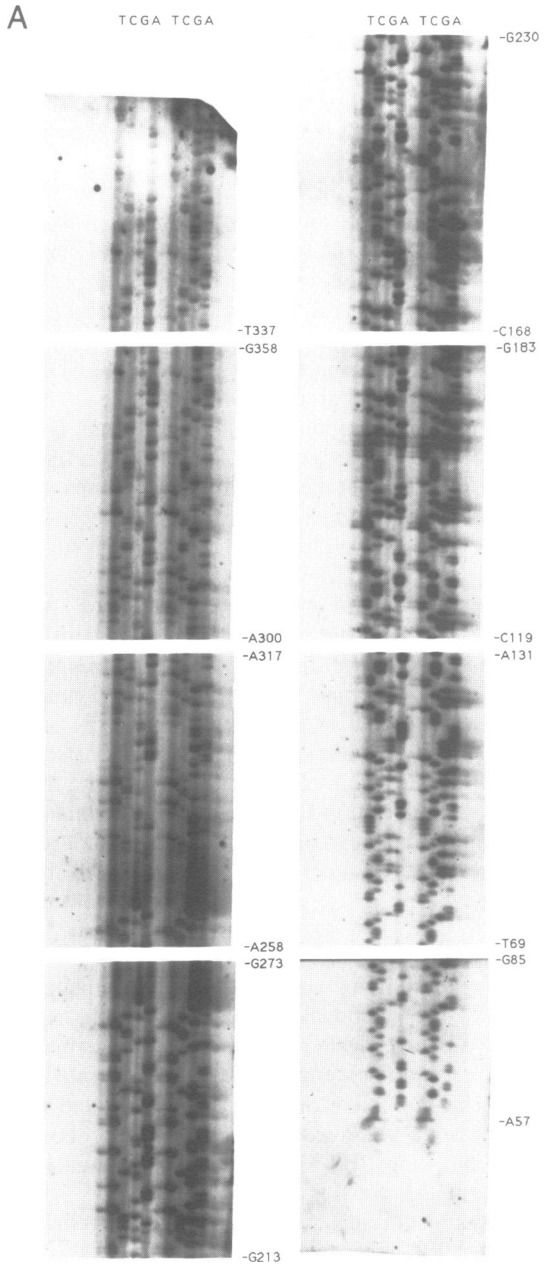
When comparing chemiluminescence detection with the oligonucleotide-AP conjugate to autoradiography with the $^{32}$P-labeled oligonucleotide, we find that the background of the chemiluminescent membrane is very low (47 amol) and indistinguishable from that of the autoradiogram (51 amol), in that respect making AP an ideal enzymatic reporter group. Comparing Figs. 1 and 2 also show, that the best chemiluminescence detection limit of 12.4 amol obtained with a 30 min CCD exposure approaches the 6.4 amol limit seen with $\gamma$-$^{32}$P-label exposured to a storage phosphor screen for the same period of time.

### Time course of oligonucleotide-AP hybridization

Before studying the binding characteristics, the kinetics of the hybridization of the oligonucleotide-AP to membrane bound plasmid DNA was investigated. The effect of the hybridization time on the chemiluminescence signal is complicated by the protein denaturing conditions during hybridization and the post-hybridization wash. Prolonged exposure of the probe and the hybrid to the blocking PBS-SB buffer at 50°C will result in significant loss in AP activity.

A series of slot blots is incubated with 2.4 nM oligonucleotide-AP in PBS-SB at 50°C and the hybridization reaction is stopped by taking slot blot samples out of the solution in 2 min intervals. Each sample is subsequently submitted to the same treatment starting with the standard 10 min wash in PBS-SB buffer at 50°C. At a probe concentration of 2.4nM, half-maximum saturation of the plasmid DNA is seen 3 min after addition of the probe solution indicating that the hybridization reaction for all practical purposes comes to completion in the course of the 15 min hybridization time given in the standard protocol.

The enzyme inactivation of oligonucleotide-AP in PBS-SB is studied in solution. Samples are assayed using p-nitrophenyl phosphate as described under Methods. The loss of activity follows a 1st-order exponential with a half-life of 81 min. About 18% of the activity is lost during the 25 min period in which the blot is submitted to these conditions during membrane development. Incubating the oligonucleotide-AP in PBS, 0.5% BSA buffer at 50°C decreases the enzyme activity by less than 3% in 25 min. Most of the loss in enzymatic activity is due to the presence of SDS in the hybridization buffer, while the inactivation due to the elevated temperature is comparably small.




B

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          10         20         30         40         50         60
AGGGTATTAA TAATGAAAAG CTGGATCCAA CCATCCTACC CCACACCTTT TAAACCAACT

          70         80         90        100        110        120
AACCTCACTC CCTCGAATTC ACTGGCCGTC GTTTTACAAC GTCGTGACTG GGAAAACCCT

         130        140        150        160        170        180
GGCGTTACCC AACTTAATCG CCTTGCAGCA CATCCCCCTT TCGCCAGCTG GCGTAATAGC

         190        200        210        220        230        240
GAAGAGGCCC GCACCGATCG CCCTTCCCAA CAGTTGCGCA GCCTGAATGG CGAATGGAAA

         250        260        270        280        290        300
TTGTAAGCGT TAATTCGACC TGCAGGGGGG GGGGGGCGCT GAGGTCTGCC TCGTGAAGAA

         310        320        330        340        350        360
GGTGTTGCTG ACTCATACCA GGCCTGAATC GCCCCATCAT CCAGCCAGAA AGTGAGGGAG

         370        380        390        400        410        420
CCACGGTTGA TGAGAGCTTT GTTGTAGGTG GACCAGTTGG TGATTTTGAA CTTTTGCTTT
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**Figure 6.** DTE sequencing blot **A**. Series of CCD images covering a 46×5.6 cm area on the blot with 2 sequencing reactions of the multiplex vector KZ2. Separation conditions: 40 cm long, 4% Long Ranger slab gel, 3 mm sharks tooth comb, 70 V/cm electric field, 4 mm/min blotting speed. Detection: hybridization in 6 nM oligonucleotide-AP, 30 min CCD exposures, 8.4×5.6 cm field of view, photonegative display **B**. DNA sequence of the pUC derived sequencing vector KZ2, the priming and probing sites are underlined.

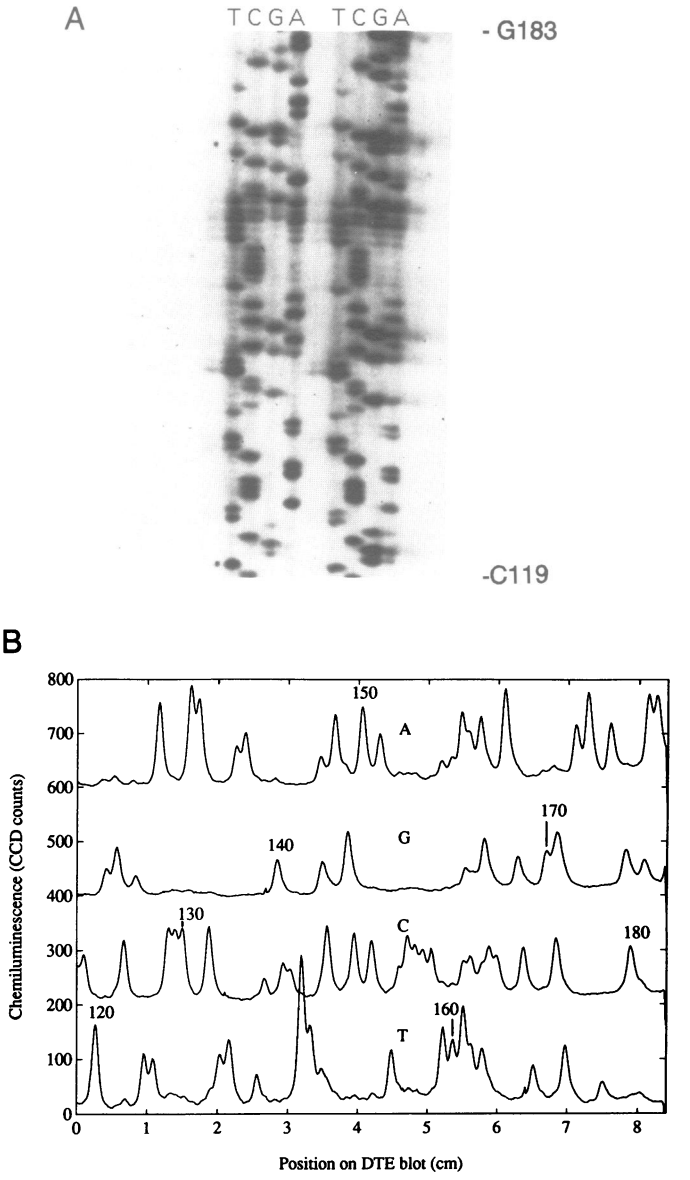


**Figure 7.** DTE sequencing blot **A**. CCD image of 8.4×5.6 cm subarea of the blot (3. frame in Fig.6) **B**. Lane set extracted from the image by averaging each lane across 2.6 mm (17 CCD elements), signal offset by 200 CCD counts for illustration.

## Binding of oligonucleotide-AP to membrane bound plasmid DNA

A series of slot blots was incubated in hybridization buffers ranging in probe concentration from 0.6 to 24 nM. The saturation of a fixed amount of bound target DNA with oligonucleotide-AP can be described by $y=B_{max}x/(K_d+x)$, where x and y are probe concentration and signal above baseline (CCD counts). Every chemiluminescence response value is the average of 4 bands containing equal amounts of bound plasmid. The apparent $K_d$ values obtained for 1024, 512, and 256 amol of target DNA are 9.0 nM, 7.9 nM, and 9.6 nM (average 8.8 nM) respectively. Fig. 3 shows the saturation isotherm, normalized for maximum binding $B_{max}$, obtained with 1024 amol of plasmid DNA. It is important to note that the degree of saturation revealed by the

binding experiment may be vastly different from the degree to which the total amount of plasmid-DNA present is hybridized. Desorption studies have shown that as little as 10% of the target DNA bound to a blotting membrane is accessible for hybridization with an oligonucleotide-AP probe[19].

The dramatic effect of the oligonucleotide-AP concentration in the hybridization buffer on the detection parameters can be seen in Fig 4 A. showing a subset of the response curves obtained in the experiment. As the signal saturates with increasing probe concentration, as illustrated in Fig. 3, a non-saturating membrane background would be expected. Somewhat surprisingly, this is not the case for the probe studied. The increase in background luminescence due to increasing probe concentration is marginal and indistinguishable from membrane-to-membrane background variations. As a consequence, the same measurement noise is observed within the range of the studied probe concentrations. The average background is 4.0 CCD counts, with an average baseline noise of 0.22 CCD counts.

The effect of increasing probe concentration on the detection limits is illustrated in Fig. 4 B. In the range of 1.2−24 nM probe concentration, the limit of detection is improved about 10-fold by the 20-fold increasing in probe concentration. By using probe concentrations above 10 nM, sub-amol detection limits can be achieved for this plasmid/probe combination. On the other hand, considering lower probe concentrations, a dramatic loss of sensitivity is observed. This is due to the proportional decrease in the rate of the hybridization reaction with decreasing probe concentration[33,34]. At the lowest concentration shown, 0.6 nM, the hybridization reaction is so slow, that the reaction does not come to completion or near completion in the course of the 15 min hybridization time. The choice of the best probe concentration will take into account both aspects, the thermodynamic saturation of the accessible binding sites as well as the kinetic limitations posed by the rate of hybridization as reflected by the two arms of the curve in Fig. 4 B. DNA bands generated by the sequencing and direct transfer blotting procedure given under Methods will typically consist of at least several tens of attomol of target DNA, an amount clearly detectable with good signal-to-noise using the oligonucleotide-AP probe in reasonable concentrations. It should be noted that the kinetic and thermodynamic parameters found in this study with the specific plasmid/probe combination can only serve as a representative example using the given protocol. They depend on experimental conditions such as probe structure, hybridization conditions and the stringency of the washing steps.

### Signal amplification using chemiluminescence enhancers

AMPPD has a rather low $\phi_{Cl} = 1.3\ 10^{-5}$[10] chemiluminescence quantum yield, meaning that the dephosphorylation of 77000 molecules is required to generate a single photon. Because of this, an increase of orders of magnitude in luminescence intensity can potentially be gained by the addition of enhancer materials to the substrate solution.

The ability of three commercially available amplifier solutions to increase the light level of AMPPD on nylon membranes was investigated. The materials dubbed Sapphire, Emerald, and Ruby (Tropix) emit fluorescence in the blue, green, and red portion of the visible spectrum when excited with light or through intermolecular energy transfer. A significant increase in signal was only seen using the Sapphire enhancer. Addition of amplifier solution increased the luminescence intensity about 1.5-fold as shown in Fig. 5. The gain in intensity is, however, accompanied by low frequency background noise, macroscopically evident as an uneven distribution of the enhancer dye on the nylon membrane. Because of this effect, the amplifier solution is not part of our standard protocol, and are not used to develop DNA sequencing blots. Fig. 5 also shows results from the same experiment carried out with AMPPD in 50 mM sodium bicarbonate/carbonate, 1 mM $MgCl_2$, pH 9.5 buffer instead of the DEA buffer. Luminescence is about 2−3-fold stronger in the DEA buffer than in the carbonate buffer, making the prior clearly the better choice.

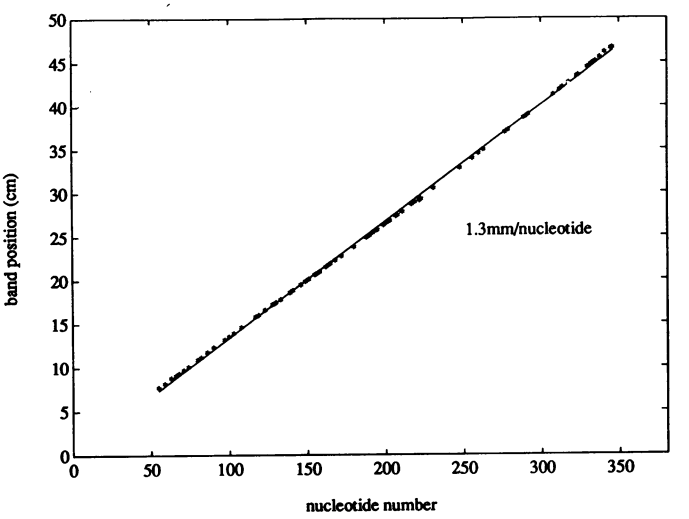


**Figure 8.** Position of bands in C-reaction lane versus fragment length on the DTE sequencing blot shown in Fig.6.

In another series, AMPPD was compared to the chloride-substituted derivative CSPD (Tropix) [18] and the ready-to-use formulation Lumi-Phos 530 (Boehringer Mannheim) [35]. Lumi-Phos 530 contains 0.33 mM AMPPD in combination with a fluorescein surfactant. In the range of plasmid DNA studied, the increase in signal of the average dot is 3-fold using CSPD and 6-fold using Lumi-Phos 530. The fluorescein derivative contained in Lumi-Phos 530 distributes evenly on the membrane surface. The increase in signal using the enhancer solution can partially be attributed to the higher CCD detector quantum efficiency at longer wavelengths. 530 nm light emitted by the Lumi-Phos preparation is detected with 31% efficiency, while the instrument efficiency at 460 nm, the emission wavelength of AMPPD in membrane based assays[16], is only 18%.

### Application to DNA sequencing

Using the sequencing, DTE blotting, and membrane development procedures under Methods a chemiluminescent blot is generated and a series of eight partially overlapping 30 min CCD exposures is taken of the blot. The frames, each representing a 8.3×5.5 cm subarea are shown in Fig. 6 A. Two adjacent images overlap by 10−20% to assure that the different portions of the sequence can easily be melded into the contiguous sequence after the sequence of the individual images is established. The sequence of the vector given in Fig. 6 B indicates the priming and hybridization sites. The smallest sequencing fragment that can be detected by hybridization assay is 57 nucleotides long. DNA sequence up to a fragment length of 350 nucleotides can be established from this blot. Considering the C-reaction lane as a

representative example, the peak heights in the 57–350 nucleotide range vary from 32 to 184 CCD counts. The baseline noise being 0.40 CCD counts, this corresponds to a signal-to-noise ratio of 80.2 for the smallest and 460 of the largest band in the C-reaction lane.

Fig. 7 shows a representative CCD frame and the lanes of one sequencing reaction extracted from that image. The lanes were averaged across 17 CCD-elements, corresponding to 2.6 mm on the blot. The bands are clearly resolved, the visible noise resulting from imperfections of the double stranded sequencing chemistry rather than from the instrument measurement, thereby meeting the primary objective of detector instrumentation for the particular task of DNA sequencing.

As has been noted earlier[2], one of the advantages of generating blots from DNA sequencing gels by run-time transfer to the membrane is the constant band-to-band spacing seen on these blots. Fig. 8 shows the position of the sequencing bands on the DTE blot to be approximately linearly related to fragment length. Deviations may occur due to compressions, the inverse wedge profile of the gel, and long term drift in the blotting speed. In DTE, the band spacing on the membrane $\Delta s_{membrane}$ is proportional to the blotting speed $v_{blotting}$ and to the ratio of the band spacing on the gel $\Delta s_{gel}$ to the migration velocity $v_{migration}$. This ratio is equal to the time interval between the elution of two adjacent bands, which is approximately constant over a wide range of fragments covering most of the separated DNA sequencing fragments.

$$\Delta s_{membrane} = \frac{\Delta s_{gel}}{v_{migration}} v_{blotting}$$

DTE gives the operator control over the band spacing on the membrane by choosing the appropriate membrane velocity, which is an important feature in the case of subsequent CCD based imaging. The CCD-detector as described under Methods samples data points every 140 $\mu$m, a relatively large sampling interval. As can be seen from Fig. 8 the average band spacing is 1.3 mm or about 9 data points per band. A more informative criterion than the number of data points per band is the sampling rate, expressed as the density of data points per standard deviation $\sigma$, where the peak is assumed to have a Gaussian profile. For the C-reaction data, the narrowest peaks, the width of which determines the required data point density, have a sampling rate of $s_0=0.26\sigma$, which is more than adequate for the sampling of Gaussian shaped bands[36]. It is important to note, that while the operator can increase the interband spacing in DTE by increasing the blotting speed, the electrophoretic resolution is not improved by this process. Loss in band resolution due to the limited resolution of the imaging optics is easily prevented by using DTE.

## CONCLUSION

This study shows the feasibility of direct digital data acquisition for nonisotopic, membrane based DNA sequencing. The approach taken is characterized by enzymatic sequencing without labeling, DTE, DNA-DNA hybridization using oligonucleotide probes, enzyme triggered, 1,2-dioxetane based chemiluminescence assay, direct digital imaging of the sequencing blots with a cooled CCD camera and computer evaluation of the digital images. The recent development in DTE instrumentation, the availability of nonisotopic, fast and sensitive detection for DNA–DNA hybridization assays and the progress of computer based sequence readers are three important steps in automating membrane based DNA sequencing. A fully automated system will require direct digital image acquisition of the blots as one step integrated into the membrane probing procedure. The study describes chemiluminescence visualization using a CCD camera as an automatable procedure for this purpose. Direct digital imaging of electrophoretic blots will allow the generation, archiving and computer evaluation of vast amounts of image information without requiring constant human attention. The concept will grow in importance as methods to generate blots become more efficient through automation and as new membrane based applications are developed. Multiplex DNA sequencing can be considered as just one example in the list of possible applications for the digital imaging of blots, for example in large scale mapping projects[37] or sequencing by hybridization[38].

The CCD camera also proved to be a powerful tool on the evaluation of membrane development procedures. Quantitative imaging was used to evaluate a protocol using biotin labeled oligonucleotides and streptavidin-AP conjugate and to compare it to the use of oligonucleotide-AP conjugates as hybridization probes. For equal amounts of probe, the biotin/streptavidin-AP based detection has the advantage of higher luminescence intensity, at the same time being limited in its ability to detect low amounts of target DNA by the nonspecific membrane binding of the biotin label and streptavidin-AP. Consequently, both reagents would have to be substituted for molecules exhibiting less nonspecific binding to the membrane to fundamentally improve the detection, for example by changing to the digoxigenin reporter group or by using direct enzyme labels. We chose to investigate the second alternative, mostly because of the technical aspects of fast, automated membrane development, 1.5 h from the start of the pre-hybridization to finishing the substrate incubation. The study illustrates how the kinetics of the hybridization reaction, enzyme inactivation and binding characteristics of the AP-labeled oligonucleotide probe influence the detection limit. Sub-attomol detection limits of membrane bound plasmid DNA are achievable with this probe and the detection of DNA sequencing bands with a signal-to-noise as high as 460 on CCD images.

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