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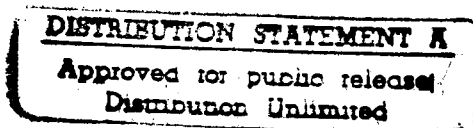
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Gene Probe Assay of Viral Nucleic Acid Using a Silicon Biosensor

BY

D. E. Bader*, Glen R. Fisher and William E. Lee

*Corresponding author

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GENE PROBE ASSAY OF VIRAL NUCLEIC ACID USING A SILICON BIOSENSOR

BY

Douglas E. Bader, Glen R. Fisher
and William E. Lee

January 1996

ABSTRACT

The use of a silicon-based biosensor for a gene probe assay is described. The target analyte, a 391 base pair DNA fragment, was mixed with a pair of probes, one labelled with biotin, the other with fluorescein, and hybridized in homogeneous solution phase. The hybridized product was separated by biotin-streptavidin mediated filtration capture and detected using a light-addressable potentiometric sensor which monitored the presence of urease conjugated (anti-fluorescein) antibodies incorporated in the hybridized product. The total assay time, including hybridization, filtration capture and potentiometric sensing was 45 - 60 min. The lower detection limit for the assay was 0.3 fmole (1.8 x 10^8 molecules) of single-stranded target DNA under low stringency conditions (Tm-22°C). The results indicate that the LAPS assay generates detection limits similar to conventional membrane-based colorimetric assays but in much less time. The LAPS assay is also less technically demanding.

EXECUTIVE SUMMARY

Title   Bader, D.E., Fisher, G.R. and Lee, W.E. "Gene Probe Assay of Viral Nucleic Acid Using a Silicon Biosensor". *DRES Suffield Memorandum No.* 1471, 1996.

Introduction   Gene probe assays are an increasingly important technology in detection and identification of biological agents, as well as medical diagnostics. Gene probe assays use discrete sequences of nucleic acid (DNA or RNA), that are complementary to distinct regions of the genetic material being analyzed. Since nucleic acid is a component of all living material, probes can be devised and used against nucleic acid from essentially all sources including viruses, bacteria, plants or animals. In addition, gene probes can be used to identify genetic sequences that have been transferred naturally or by human design into foreign vehicles, for example, toxin genes cloned into innocuous organisms or infectious nucleic acid contained within man-made microcapsules.

Gene probe assays are conventionally performed as mixed-phase assays whereby the target DNA is bound to the solid phase and the probe hybridizes to the target in solution phase. Mixed-phase assays, while being very sensitive, tend to be slow because extra time is required to bind target to the solid phase and block the solid phase following target binding. In addition, mixed-phase reactions are not as kinetically favoured as solution-phase reactions with reaction rates being up to 10 times slower. These types of assays are also more technically demanding and cumbersome. Any improvements in reducing assay time and complexity, without compromising sensitivity, would be beneficial to the utility of gene probe assays for identification purposes. This preliminary study describes the development of a solution-phase assay format using a commercially available biosensor-based detection apparatus (LAPS) and compares it to a conventional mixed-phase approach in terms of assay sensitivity, speed and ease of use. A DNA fragment of the nucleocapsid protein gene of Newcastle disease virus was used as the target DNA.

Results   The biosensor-based assay was characterized as a function of temperature, hybridization time, and probe concentration. The lower limit of detection was found to be about 0.3 fmol of target DNA (1.8×10^8 molecules) under low stringency conditions (Tm-22°C). Total assay time including hybridization and potentiometric sensing was about 45-60 min.

Significance of Results   Comparative analysis of these results with those obtained for a mixed-phase membrane-based assay using the same probe/target system suggests that the biosensor assay generates equivalent detection limits but in much less time. In addition, the biosensor-based assay is much less technically demanding.

The LAP sensor apparatus has been demonstrated in previous DRES studies to be effective as an analytical system to carry out immunoassays for protein (toxins), virus, bacteria, and anti-cholinesterase organophosphorous compounds. The immuno-based LAPS system was recently adopted as one of two identification technologies for integration into the Canadian Integrated Biological Agent Detection System (CIBADS) which performed well during recent field trials undertaken at Dugway Proving Grounds (USA) in September 1995. This study demonstrates the expanded capability of the LAP sensor to carry out rapid, sensitive gene probe assays with the potential for integration into the CIBADS.

Future Goals   More studies are required to determine whether the gene probe-based LAPS assay performs as well as membrane-based assays under more stringent hybridization conditions. Higher stringencies may be required in order to improve specificity of the probe/target interaction.

There is good potential for the gene probe-based LAPS assay to be integrated into CIBADS and tested under field trial conditions, however, there are other commercially available biosensor systems that could offer significant advantages over the LAPS. For example, surface plasmon resonance or SPR, does not require secondary detection of probe/target analyte, thereby reducing detection time as well as reducing reagents and consumables. Gene probes will continue to play an important role in identification strategies being developed for the CF.

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INTRODUCTION

Gene probe assays are an increasingly important technology in detection and identification of biological agents, as well as medical diagnostics, and can be used as an alternative to or as a complement to immunoassay analysis. Immunoassays are used to detect the presence of biological macromolecules, and to do so employ antibodies which are directed at specific chemical features of the analyte molecule or macromolecular assembly. Gene probe assays on the other hand use discrete sequences of nucleic acid (DNA or RNA) that are complementary to distinct regions of the genetic material being analyzed. Since nucleic acid is a component of all living material, probes can be devised and used against nucleic acid from essentially all sources including viruses, bacteria, plants or animals.

Gene probe assays are conventionally performed as mixed-phase assays whereby the target DNA is bound to the solid phase and the probe hybridizes to the target in solution phase. We have previously developed two mixed-phase gene probe assays, namely, a membrane-based assay [1] and a microtiter plate assay [2]. Mixed-phase assays tend to be slower than solution-phase hybridization assays because extra time is required to bind target to the solid phase and block the solid phase following target binding. In addition mixed-phase reactions are not as kinetically favoured as solution-phase reactions. Reaction rates for mixed-phase reactions have been demonstrated to be up to 10 times slower [3]. The purpose of this work was to develop a solution-phase, biosensor-based system that could provide quantitative analysis more rapidly than conventional approaches.

In this study, we describe the development of a dual gene probe assay for Newcastle disease virus (NDV). NDV is an avian paramyxovirus. The genome is comprised of a single, non-segmented molecule of negative sense RNA, approximately 15,000 bases in length. In this work the B1 vaccine strain of NDV, a commercially available poultry vaccine, was employed. As a model, this strain of NDV has a number of advantages. It has been approved by Agriculture Canada for release into the air and has been identified for use as a

BW viral simulant in field trials [4]. NDV has been characterized by a number of immunoassay studies [5, 6, 7] and served as a virus analyte in the development of new biosensor technologies [8, 9, 10].

The dual probe assay is shown in schematic in Figure 1. The target for the assay was a 391 base pair DNA fragment of the major nucleocapsid protein gene of NDV which was prepared by reverse-transcription polymerase chain reaction amplification (RT-PCR) of purified genomic NDV B1 RNA. The gene probes used in this assay were single-stranded oligonucleotide probes (20-23 bases) that were designed based on sequence information for NDV strain D26 [11] and were nested within the 391 bp target. One of the probes was 5' end-labelled with biotin and served as a non-specific anchoring probe. The other probe was 5' end-labelled with fluorescein and served as the "NDV specific" probe based on sequence alignment analysis of NP gene sequences from representative viruses in the Paramyxoviridae family [12].

The detection apparatus used was a silicon-based light addressable potentiometric (LAP) sensor [13]. The probes and double-stranded target were mixed together, denatured and hybridized in solution. After hybridization, streptavidin was added. The reaction mixture was filtered through a biotinylated nitrocellulose membrane thus immobilizing the hybridization complex. A solution of anti-fluorescein urease conjugate, was filtered over the complex and excess reagents were washed away. The membrane containing the final product, hybridized target-probe/antibody-enzyme, was inserted into the reader compartment of the LAP sensor which held a solution of urea substrate. A plunger pressed the membrane against the surface of the pH-sensitive silicon sensor. Catalytic hydrolysis of urea resulted in a pH change on the membrane. The presence of target DNA on the membrane was expressed as change in output signal of the pH-sensitive sensor.

In this work we have attempted to characterize the interaction of probe and target as a function of temperature, hybridization time, and probe concentration. The lower limit of detection was found to be about 0.3 fmol (1.8×10^8 molecules). Total assay time including hybridization and potentiometric sensing was about 45-60 min.

MATERIALS AND METHODS

Reagents   Bovine serum albumin (BSA), ethylene diamine tetra-acetic acid (EDTA), sodium dihydrogen phosphate, Triton X-100, Tween-20 and urea, were obtained from Sigma Chemical Co. (St Louis, MO) and used without any further purification. Streptavidin was obtained from Scripps Laboratories (San Diego, CA). All water used in this study was either triple distilled, deionized water which was made sterile by autoclaving or nuclease-free water from Fisher-Promega Scientific (Edmonton, AB).

RT-PCR primers and oligonucleotide gene probes were synthesized by the Regional DNA Synthesis Laboratory of the University of Calgary (Calgary, AB).

RT-PCR primers

| NDVNP-PR7 | 5' OH-ctg cca aaa tgt ctt ctg tat-OH 3' | 21 bases |
| NDVNP-PR8 | 5' OH-aat ctc tgt gct ctc tct tca-OH 3' | 21 bases |

Gene Probes

| NDVNP-PB4 | 5' fluorescein-gct cct cgc ggc tca gac tc-OH 3' | 20 bases |
| NDVNP-PB8 | 5' biotin-tag cga gga tgc caa caa acc ac-OH 3' | 23 bases |

Oligo^{TM} Primer Analysis Software program version 4.1 from National Biosciences (Plymouth, MN) and PC Gene version 6.5 software from Intelligenetics Incorporated (Mountainview, CA) were used for primer and gene probe design based on sequence information for NDV strain D26 [11]. The RT-PCR primers were designed to amplify a 391 bp fragment of the NDV major nucleocapsid protein gene. The gene probes were designed to bind within the 391 bp sequence and to have similar thermal stabilities (Table I).

Anti-fluorescein urease conjugate and nitrocellulose membrane filters (0.44 μm pore size) coated with biotinylated bovine serum albumin were purchased from Molecular Devices Corporation (Sunnyvale, CA).

Apparatus   Reverse transcription-polymerase chain reaction was carried out using a Perkin Elmer-Applied Biosystems thermal cycler (Mississauga, ON). Horizontal agarose gel electrophoresis was performed using power packs from BioRad (Mississauga, ON) and electrophoresis apparatus from Gibco-BRL (Burlington, ON). UV illumination and visualization of ethidium bromide stained agarose gels were performed on a Fotodyne UV light box from Bio/Can Scientific (Mississauga, ON). The sensing apparatus for the assay was a commercially available LAP sensor, marketed under the name Threshold Unit. It was purchased from Molecular Devices (Sunnyvale, CA). The instrument was controlled by an IBM PS/2 model 30 microcomputer and custom designed software, supplied by Molecular Devices. The assay system was capable of processing (filtration, potentiometric sensing) eight samples simultaneously.

Buffers   *Hybridization buffer* (2x)

60 mM sodium phosphate (pH 7.4), 6 mM EDTA, 900 mM NaCl, and 0.5% Triton X-100. The hybridization buffer was filter-sterilized before use.

Wash solution

10 mM sodium phosphate buffer (pH 6.5), 1 mM EDTA, 150 mM NaCl and 0.05% Tween-20 detergent. The wash solution was filter-sterilized before use.

Assay buffer

10 mM sodium phosphate buffer (pH 7.0), 1 mM EDTA, 150 mM NaCl, 0.05% Tween-20 detergent and 0.1% BSA. The assay buffer was filter-sterilized before use.

Substrate solution

10 mM sodium phosphate buffer (pH 6.5), 1 mM EDTA, 150 mM NaCl, 0.05% Tween-20 detergent and 100 mM urea.

Methods *Preparation of Target DNA by RT-PCR*

Target DNA was prepared by RT-PCR of purified NDV B1 genomic RNA based on procedures described elsewhere [12] with two modifications. First, the primers used in this study were NDVNP-PR7/8 which generated a 391 bp amplicon (as opposed to the primers used in the other study which generated a 673 bp amplicon). Second, a different PCR cycling program was used in this study: 94°C for 5 min; 94°C for 1 min + 54°C for 2 min + 72°C for 3 min (30 cycles); 72°C for 7 min.

Assay Procedure

Double-stranded target DNA was combined with excess probe in hybridization buffer (probes were always used in equimolar amounts) and made to 1x buffer strength with water to a total volume of 60 μL. This mixture was denatured at 100°C for 15 min to separate double-stranded target DNA into single strands and then cooled to 10°C for 5 min. Hybridization was carried out by heating the mixture to the required hybridization temperature for a specified time and then cooling rapidly to 4-10°C. A 50 μL aliquot of hybridized product was added to 100 μL of assay buffer containing 1 μg streptavidin and thoroughly mixed. A 135 μL aliquot of the mixture was filtered under partial vacuum through a nitrocellulose membrane pre-coated with biotinylated BSA. After filtration the membrane was washed with 1 mL of wash buffer. The vacuum was reduced and 100 μL of anti-fluorescein urease conjugate was slow-filtered through the membrane over 15 min. The membrane was washed with 2 mL of wash buffer to remove excess conjugate. The membrane stick was removed from the filter apparatus and inserted into the reader compartment of the sensor. The output signal from the sensor was recorded by the computer as μV/sec.

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RESULTS

In the dual probe assay format, the biotin-labelled and fluorescein-labelled probes were hybridized in equimolar concentrations to target DNA in a homogeneous phase reaction. Gene probes were always present in excess over target, ranging from 20-fold to greater than 1000-fold.

The formation of the hybridization complex (probe and target) was determined as a function of time. The results for two probe concentrations are presented in Figure 2. The lower probe concentration (5 nM) yielded a maximum signal after about 15 min while the higher probe concentration (20 nM) yielded a maximum signal after about 7.5 min. Both curves showed a decrease in signal after the maximum signal was reached. This decrease in signal is likely due to "snap back", whereby initially, the formation of the probe/target complex is the kinetically favoured product, however, as the hybridization reaction proceeds further, the probes are displaced by the more thermodynamically stable complementary strand of the target molecule which is present in the target DNA sample.

The effect of probe/target complex formation with hybridization temperature is shown in Figure 3. As the temperature increases, the amount of probe/target complex decreases. This same experiment was carried out at higher probe concentrations. While the output signals were found to be higher, the same trend was observed.

The signal-to-background ratio was measured over a range of probe concentrations from 2.5 nM to 40 nM using a constant amount of target (Figure 4). The increase in signal-to-background ratio was due to the increase in signal of the target-containing sample, since the background signals did not increase proportionately. The maximum ratio was obtained at an equimolar probe concentration of 20 nM.

A standard curve of the target DNA across a target range of 0 to 25 fmol/well is shown in Figure 5. There was a monotonic increase in signal in response to increasing amounts of target DNA.

The lower detection limit (LDL) was determined for the assay by testing replicate samples of the target over a lower concentration range (0 to 2.5 fmol/ well). The lower detection limit was taken to be the lowest amount of target which provided a signal greater than the background plus two standard deviations. The results are shown in Figure 6. The lower detection limit was found to be 0.3 fmol of target DNA or about 1.8×10^8 molecules in the presence of 300 fmol or 1.8×10^{11} molecules of each probe (5 nM) at 58°C hybridization temperature (stringency = Tm-22°C). Under these conditions, the maximum formation of hybridization product was attained in 15 min. Assay time, including hybridization, filtration capture and potentiometric sensing was about 60 min. The coefficients of variation (mean/sd) were about 10 - 12%.

DISCUSSION

This work describes the use of a silicon-based biosensor for a gene probe assay. A comparison of the results obtained in this study versus those obtained for a membrane-based gene probe assay is presented in Table II. Generally speaking, under the assay parameters described, the LAPS assay generated similar detection limits to the membrane assay but in a considerably shorter time frame. However, these comparisons are based on different hybridization stringencies and probe concentrations, which in turn can have an effect on detection limits and hybridization reaction times. For example, in this study, we demonstrated that an increase in the stringency reduced signal output while an increase in the probe concentration (from 5 nM to 20 nM) increased the signal output and reduced the hybridization time.

We have previously shown the LAP sensor to be effective as an analytical system to carry out immunoassays for protein [15], virus [8], bacteria [16] and anti-cholinesterase organophosphorous compounds [17]. We have now expanded the capability of the LAP sensor to carry out rapid, sensitive gene probe assays for viruses. Gene probes will continue to play an important role

in BW agent identification because of their unique ability to detect and identify conventional BW agents (viruses, bacteria, rickettsias etc.) as well as modified or novel threat agents, for example, man-made vesicles containing infectious nucleic acid or innocuous organisms containing cloned toxin genes. In addition, gene probes can be used in nucleic acid amplification reactions, thereby allowing amplification and detection of very limited quantities of target analyte from living, non-living and living but non-culturable organisms.

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Table I

Tm Values for the Gene Probes Used in this Study[1]

Conditions	NDVNP-PB4	NDVNP-PB8
Salt conc = 480 mM Probe conc = 5000 pM	79.8°C	80.4°C
Salt conc = 480 mM Probe conc = 20000 pM	82.1°C	82.4°C

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[1]Estimates of the melting temperatures (Tm) were calculated by "Nearest-Neighbour Method" using Oligo Primer Analysis Software program version 4.1 from National Biosciences [14].

Table II

Gene Probe Assay Comparison

	LAPS Assay	Membrane Assay[2]
probe conc	5 nM	30 nM
Na+ concentration	480 mM	16.5 mM
Tm	79.8°C	58.4°C
Ti	58°C	45°C
Stringency	Tm-22°C	Tm-13.4°C
Hybridization time	15 min	1 h
Assay time	1 h	6 h
Detection system	potentiometric	colorimetric
Lower detection limit	10^8 molecules of a 391 bp DNA fragment from the NDV NP gene	10^8 molecules of a 673 bp DNA fragment from the NDV NP gene

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[2]The data in this column has been taken from [1].
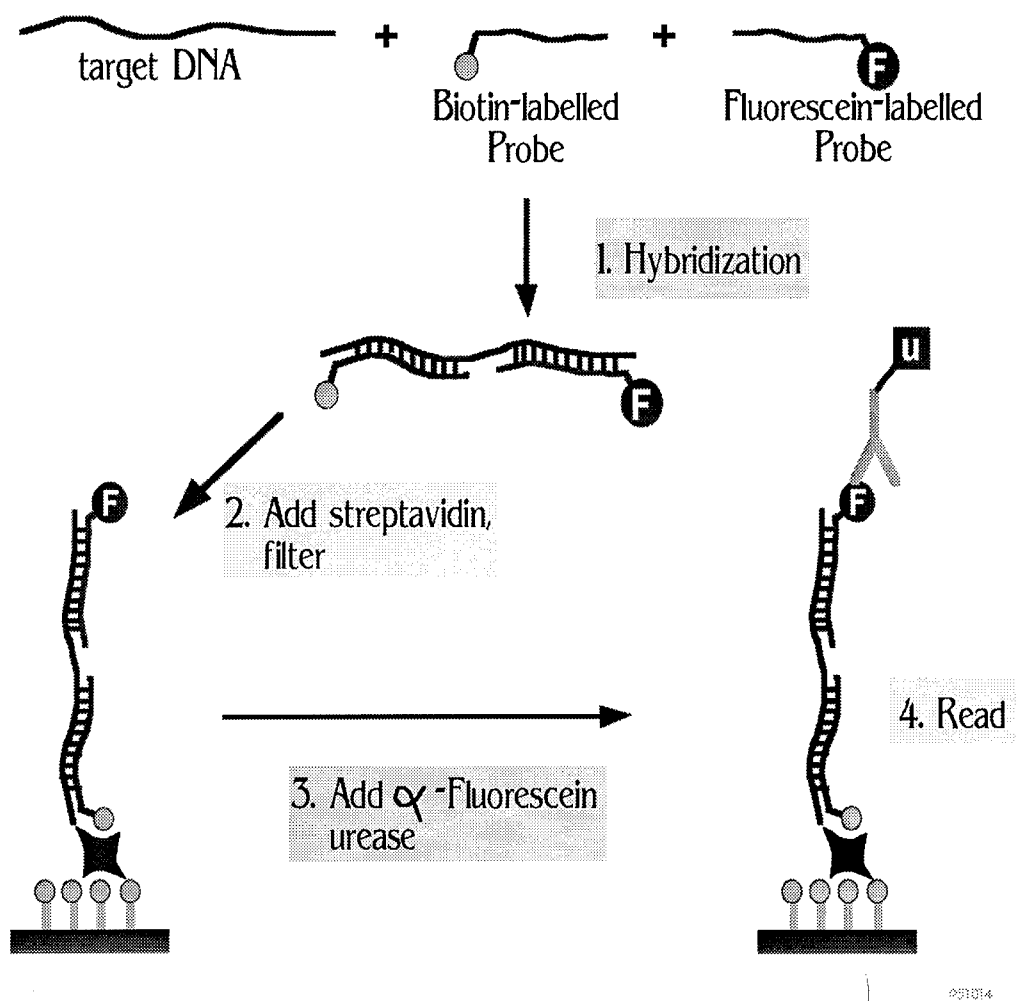
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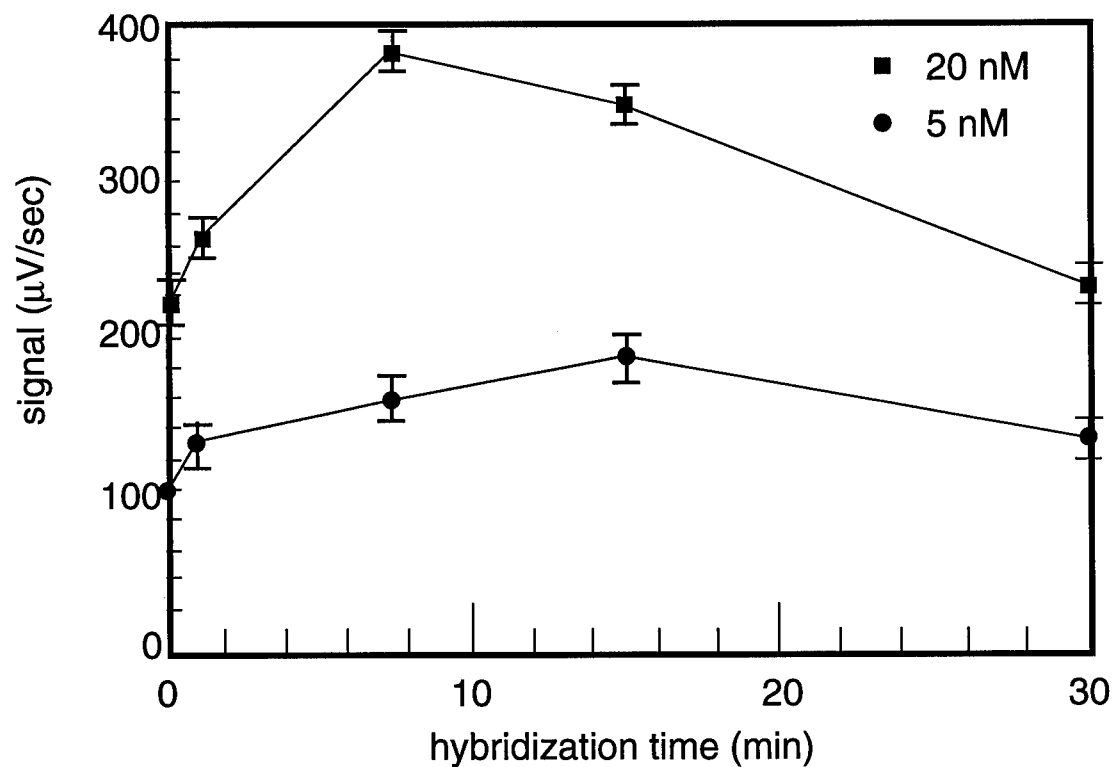
Schematic diagram of the dual probe LAPS assay.

Figure 2

Formation of probe-target complex as a function of hybridization time using a hybridization temperature of 58°C and 10 fmol of target DNA per well. Biotin-labelled and fluorescein-labelled probes used in equimolar amounts. Stringency = Tm-22°C.
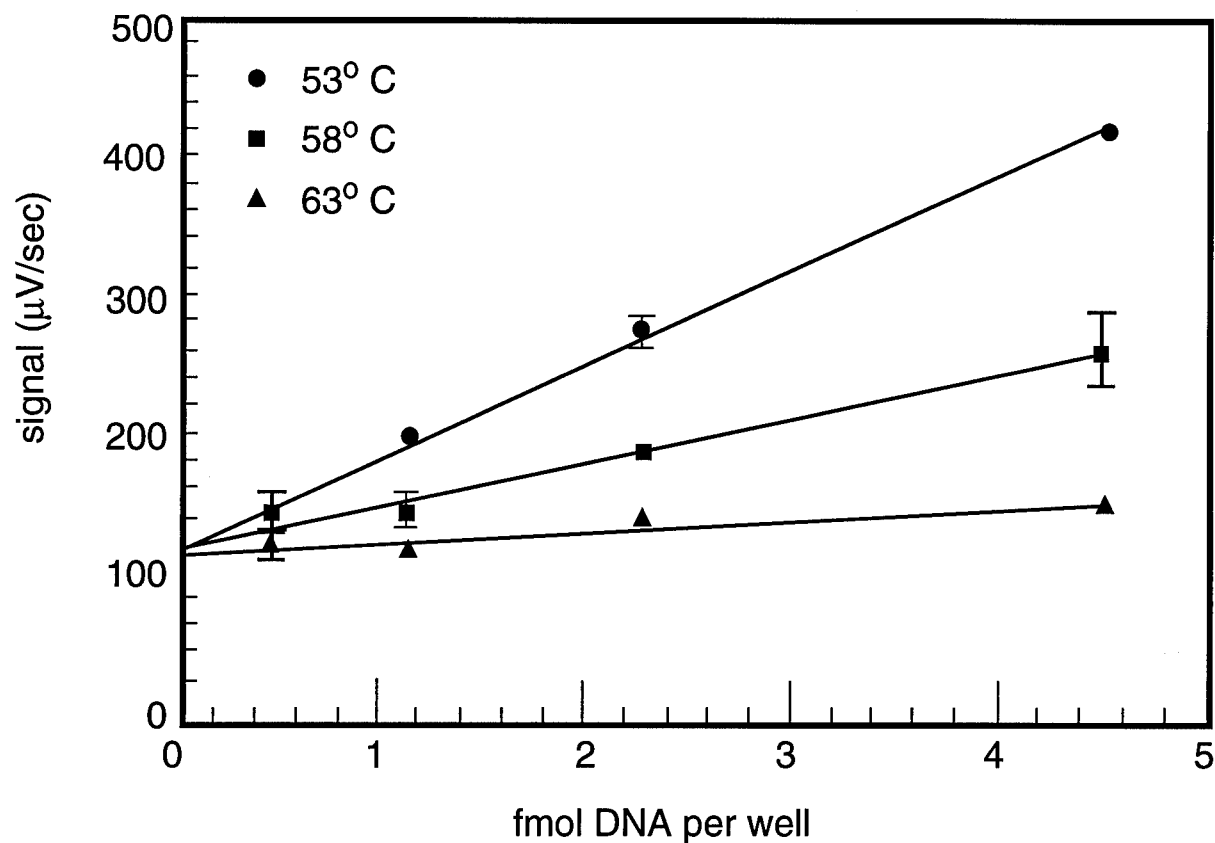
Figure 3

Effect of hybridization temperature on signal output at 5 nM equimolar probe concentrations.
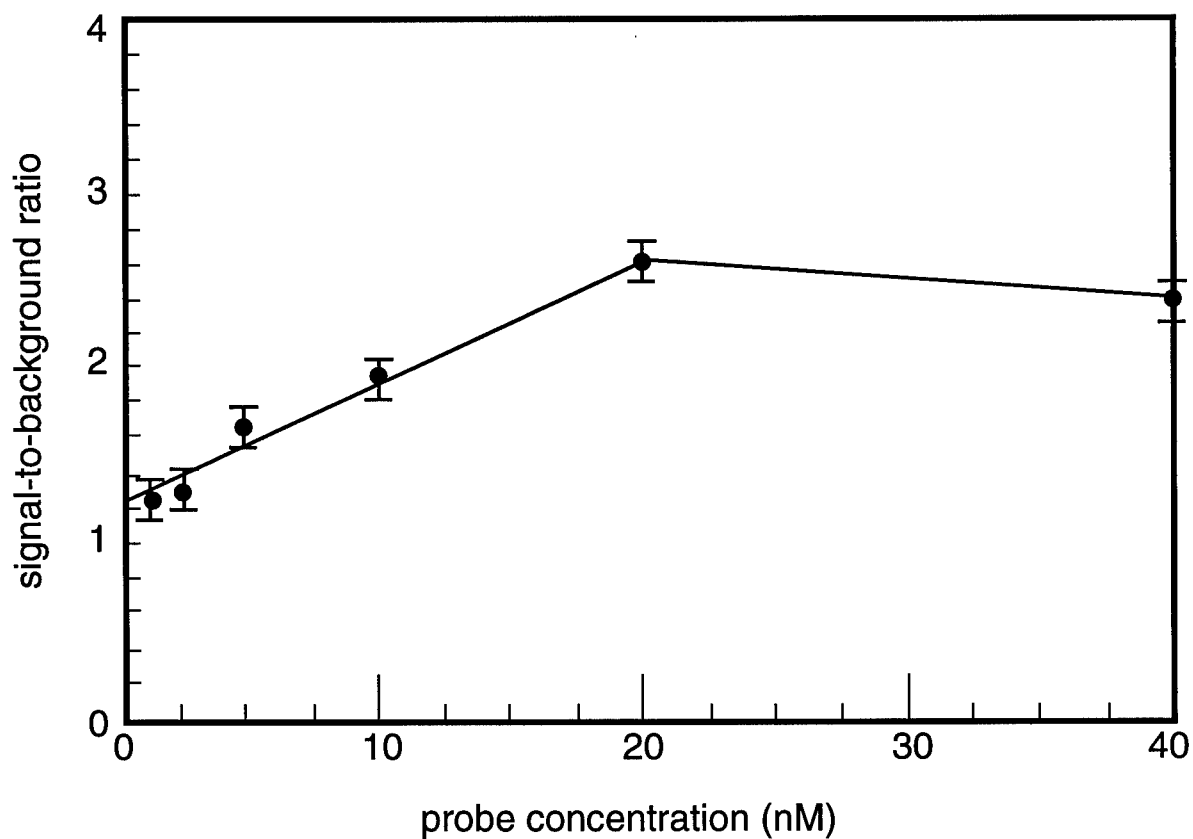
Figure 4

Effect of probe concentration on signal/background ratio using a hybridization temperature of 58°C and 10 fmol of target DNA per well. Stringency = Tm-22°C.
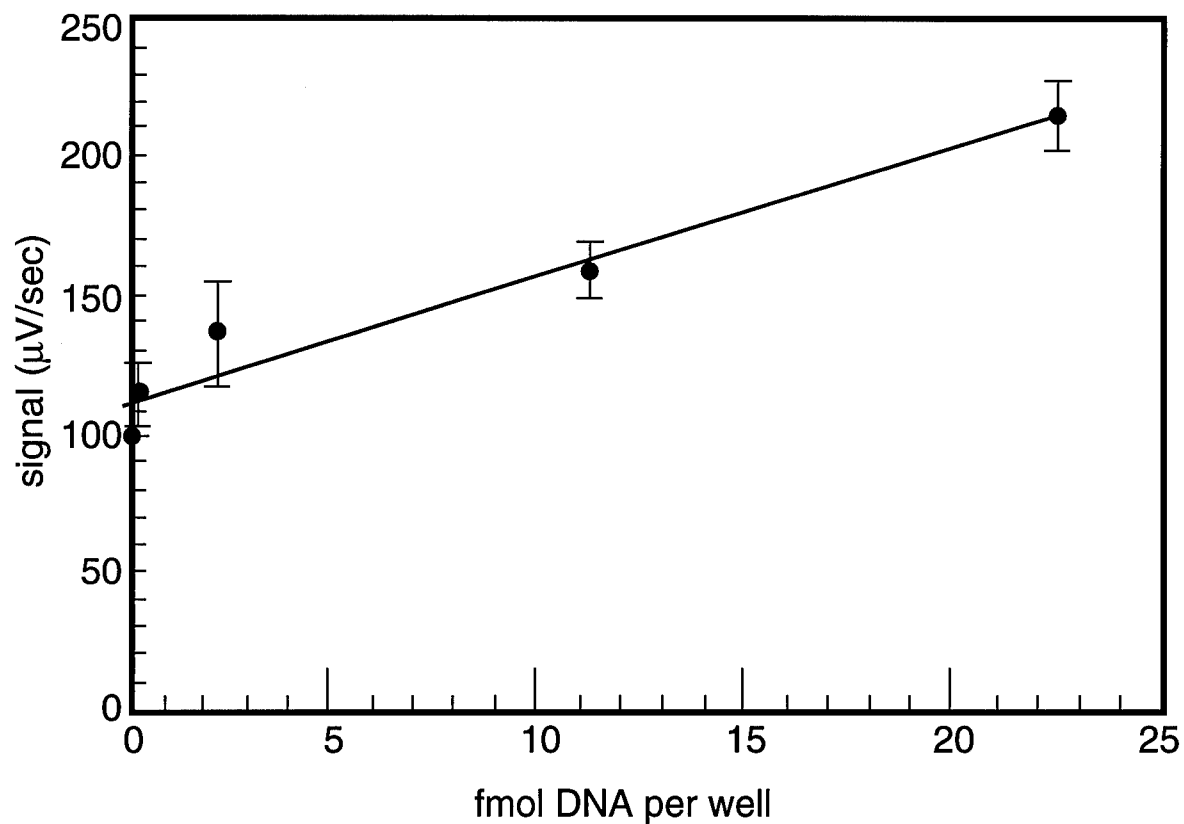
Figure 5

Response curve for a target range of 0 to 25 fmols of DNA per well using a hybridization

temperature of 58°C and an equimolar probe concentration of 5 nM. Stringency = Tm-22°C.
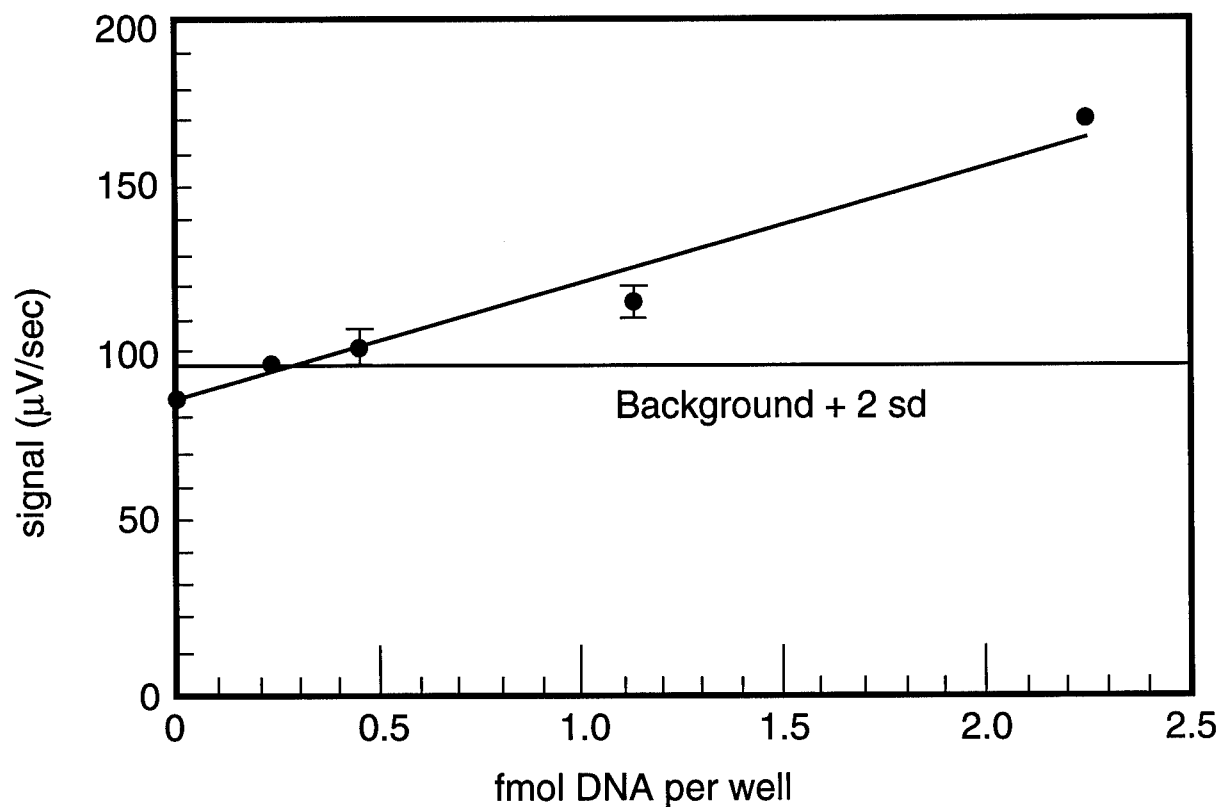
Figure 6

Determination of the lower detection limit for target DNA in the range of 0 to 2.5 fmol per well

using a hybridization temperature of 58°C and an equimolar probe concentration of 5 nM.

Stringency = Tm-22°C.

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The use of a silicon-based biosensor for a gene probe assay is described. The target analyte, a 391 base pair DNA fragment, was mixed with a pair of probes, one labelled with biotin, the other with fluorescein, and hybridized in homogeneous solution phase. The hybridized product was separated by biotin-streptavidin mediated filtration capture and detected using a light-addressable potentiometric sensor which monitored the presence of urease conjugated (anti-fluorescein) antibodies incorporated in the hybridized product. The total assay time, including hybridization, filtration capture and potentiometric sensing was 45 - 60 min. The lower detection limit for the assay was 0.3 fmole (1.8×10^8 molecules) of single-stranded target DNA under low stringency conditions (Tm-22°C). The results indicate that the LAPS assay generates detection limits similar to conventional membrane-based colorimetric assays but in much less time. The LAPS assay is also less technically demanding.

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Identification

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