

## SIMPLE DETECTION METHOD OF <sup>32</sup>P- AND <sup>3</sup>H-LABELED DNA PROBES

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**ABSTRACT.** The advent of DNA amplification methods has enabled applications of DNA probes to encompass laboratory diagnoses of microbial, genetic and neoplastic diseases. At the same time, the need for a simple and rapid detection method for amplified DNA has increased. We employed <sup>32</sup>P-labeled oligonucleotides in the detection of amplified DNA sequences, using a solution hybridization method and the microtitration plate format. We also measured the incorporation of <sup>3</sup>H-labeled nucleotides in a minisequencing assay using the same assay format and a Microbeta™ liquid scintillation counter. We amplified the ΔF508 mutation site, which is present in about 70% of cystic fibrosis chromosomes, using the polymerase chain reaction. The presence or absence of normal and mutant alleles was then determined using allele-specific oligonucleotide probes or primer-guided incorporation of specific <sup>3</sup>H-nucleotide. As a result, the sample could be interpreted as normal, carrier or mutant for the ΔF508 mutation. This type of assay makes the simple and rapid detection of mutations possible and can be performed conveniently in the microtitration plate format.

### INTRODUCTION

The use of various nucleic-acid hybridization methods in research and diagnoses of many diseases has increased tremendously during the past few years. These hybridization methods have been used to diagnose neoplastic, genetic and infectious diseases. Nucleic acid hybridization methods are also widely employed in molecular biology laboratories (Lowe 1986).

Such methods, *e.g.*, dot-blot, Southern and Northern hybridizations, include relatively complex sample treatment and overall hands-on procedures. Hybridization kinetics are slow but the sensitivities can be high. Hybridization in solution (Syvänen, Laaksonen & Söderlund 1986) has certain advantages for automation, including a simple treatment and sample assay, rapid kinetics in solution and high sensitivity when linked to an amplification reaction.

Isotopic labels are still widely employed in molecular biology laboratories, along with various hybridization methods. Among the labels used, <sup>32</sup>P offers high sensitivity, but has a short half-life ( $t_{1/2} = 14.3$  d), whereas <sup>3</sup>H does not introduce a significant health hazard and it has a much longer decay time ( $t_{1/2} = 12.4$  yr). The detection of <sup>3</sup>H-labeled nucleotides after an amplification step can be done readily with scintillation counters. However, conventional scintillation counting can be time-consuming, especially if large numbers of samples are to be tested.

Our goal was to develop simple and rapid hybridization assays by focusing on the assay format (Iitiä *et al.* 1991) and on the detection of isotopically labeled nucleotides. We developed assays for detection of the cystic fibrosis (CF) ΔF508 mutation, the three base-pair deletion, which is present in about 70% of the CF chromosomes in Caucasian populations. One assay is based on <sup>32</sup>P-labeled oligonucleotides and a solution hybridization method performed in the microtitration plate format. After hybridization, the solid phase-bound hybrids were quantified directly from microtitration strips using the Microbeta™ counter. In the minisequencing assay (Syvänen *et al.* 1990), the detection is based on an enzymatic reaction where the DNA polymerase, a specific primer and one labeled nucleotide are needed. The polymerase chain reaction (PCR)-amplified DNA is bound to microtitration strips and DNA polymerase extends the DNA chain with one single nucleotide, which complements the nucleotide at the specific site. The bound hybrids are then measured using a Microbeta™ counter.

## METHODS

**Allele-Specific Oligonucleotide (ASO) Method Using <sup>32</sup>P-Labeled Probes**

A 3-mm filter-paper disk was cut from dried blood spots using a DELFIA® Plate Punch, and placed in a 500- $\mu$ l Eppendorf tube. DNA was liberated by incubating the disks in 50  $\mu$ l of 10 mM NaOH, 1% Nonidet P-40 (Sigma, St. Louis, Missouri, USA) at 100°C for 10 min. After neutralization, the samples were centrifuged, and a sample was taken into the PCR reaction from the supernatant. The oligonucleotides were synthesized using a Gene Assembler Plus synthesizer (Pharmacia LKB Biotechnology, Uppsala, Sweden) and phosphoramidate chemistry. For biotinylation, one modC (Sund *et al.* 1988) was introduced to the 5' end of the specific (5'-TCTCAGTTTCTGGATTAT) oligonucleotide. The allele-specific probes (20 pmol) were labeled with 40 pmol of  $\gamma$ -ATP, >5000 Ci/mmol and 15U of T4 polynucleotide kinase (Amersham International plc, Buckinghamshire, UK). After completion of the reaction, the oligos were purified using Nick gel filtration columns (Pharmacia). The mutation-specific probe (5'-AATATCAT---TGGTGT) had a specific activity of  $1.3 \times 10^8$  cpm  $\mu$ g<sup>-1</sup>, and the normal specific probe (5'-TATCATCTTTGGTG),  $1.4 \times 10^8$  cpm  $\mu$ g<sup>-1</sup>.

The PCR reaction was carried out in a Perkin-Elmer Cetus Thermocycler using the program 95°C, 50 s  $\rightarrow$  53°C, 2 min  $\rightarrow$  73°C, 2 min, for 28 cycles. The final reaction mixture contained 10 mM Tris-HCl pH 8.5, 0.2% Nonidet P-40, 5.0 mM MgCl<sub>2</sub>, 200  $\mu$ M each dNTP, 0.1  $\mu$ g ml<sup>-1</sup> bovine serum albumin and 50 mM NaCl. We used 20  $\mu$ l of pretreated sample material in a final volume of 100  $\mu$ l.

The solution was hybridized for 1 h at 35°C in uncoated microtitration strips (Nunc, Roskilde, Denmark) with oligonucleotide probes (1 ng/well) in a hybridization solution (100  $\mu$ l, containing 0.5 M NaCl, 25 mM Tris-HCl pH 7.75, 0.005% Tween®-40, 0.25% BSA, 0.025% bovine globulin, 10  $\mu$ M DTPA and 0.025% NaN<sub>3</sub>), and 10  $\mu$ l of denatured PCR product. The hybrids were collected onto a solid phase by transferring the solution into streptavidin-coated microtitration strips (Wallac Oy), which were incubated for 1 h at room temperature (RT). After washing at RT (DELFIA® Wash Solution), the plates were placed in a cassette and the Cerenkov radiation measured (Fig. 1); alternatively, a piece of solid scintillant (Meltilex™, Wallac Oy) was added to the dry wells prior to the measurement to enhance the signal (Table 1). The Microbeta™ scintillation counter automatically corrects for the crosstalk signal both using a plate cassette and a patented software program.

TABLE 1. Representative Results for <sup>32</sup>P-Labeled Probes of Patient and Control Samples Using the ASO Method (cpm)

	Samples			Controls		
	Normal	Carrier	Mutant	Normal	Carrier	Mutant
Normal probes	3336	279	52	2452	1030	94
Deletion probes	51	720	9037	40	1540	2742
Ratio*	65	0.38	0.006	61	0.67	0.03

\*The ratio of the specific signal to the cross-reaction signal

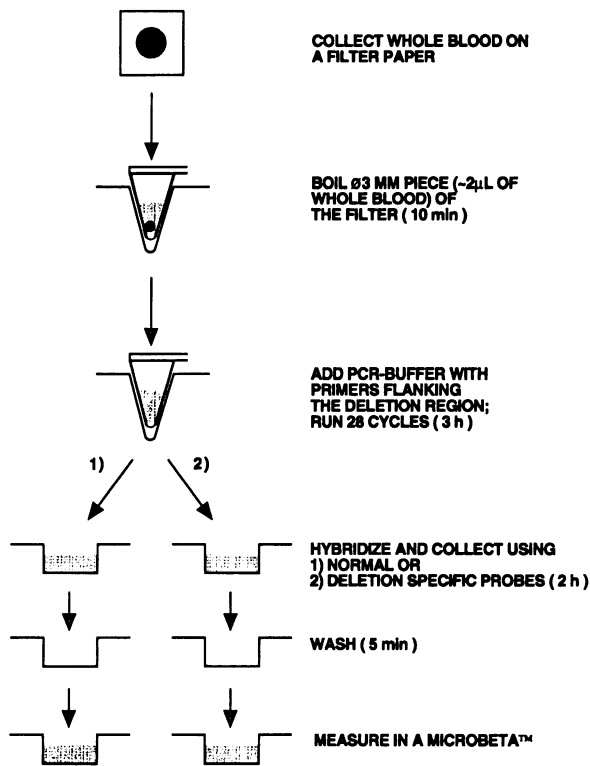


Fig. 1. Principle of ASO hybridization assay using <sup>32</sup>P-labeled probes

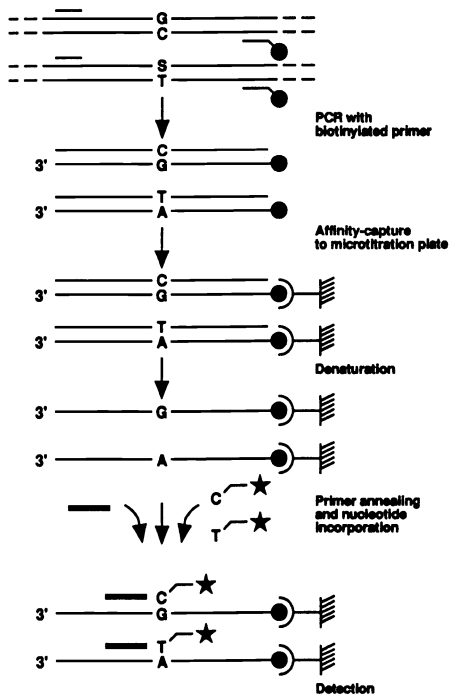


Fig. 2. Principle of solid-phase minisequencing assay for the detection of point mutations. Modified from Syvänen *et al.* (1990).

### Minisequencing Assay Using $^3\text{H}$ -Labeled Nucleotides

After PCR amplification reaction including biotinylated primers, the hybrids were collected onto streptavidin-coated microtitration plates for 1 h at 25°C (Fig. 2). After denaturation, new primers were added into the wells (Wallac Oy) and incubated for 30 min at 35°C. The plates were washed, and labeled nucleotides and DNA polymerase were added and incubated for 20 min at 47°C. After the polymerase reaction, the wells were washed and 200  $\mu\text{l}$  of Optiphase HiSafe™ 2 scintillation cocktail (Wallac Oy) were added. The plates were then measured in the Microbeta™, as above.

## RESULTS

### ASO Method

Both probes had their optimal hybridization temperature at 30–35°C (data not shown). When synthetic oligonucleotide targets were tested,  $\sim 6 \times 10^7$  to  $1 \times 10^8$  target molecules could be detected. The dynamic range of the assay was from  $1\text{--}3 \times 10^8$  to  $1 \times 10^{10}$  molecules per well, giving cpm values from 177 to 21,487 (Fig. 3). When blood specimens and amplified DNA control specimens

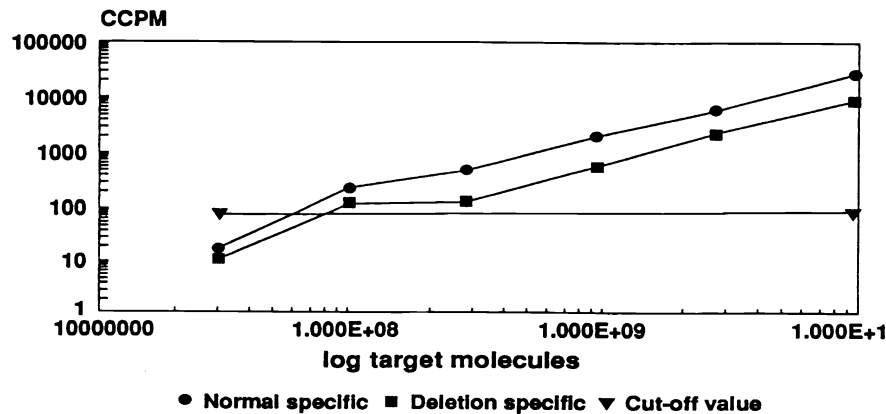


Fig. 3. Quantification of  $^{32}\text{P}$ -labeled oligonucleotides with Microbeta. ● = Normal-specific; ■ = Deletion-specific; ▼ = Cut-off value

were tested, the ratios (normal probe/deletion probe) for normal samples were above 10, for carriers between 0.2 and 1.0, and for mutant samples below 0.1 (Table 1). In addition, the measuring efficiency of  $^{32}\text{P}$  with Microbeta™ was equal to that of standard liquid scintillation counters when measured with a scintillant in 1450-401 plates. With standard, clear microtitration plates, *i.e.*, “free plates”, the efficiency was >90% using Optiphase HiSafe™ 2 cocktail (Wallac Oy). We selected relatively short oligonucleotides (14 mers) to detect the alleles from PCR-amplified material. We found that short oligonucleotides improve the assay specificity compared to longer oligonucleotide probes, and allow lower washing and hybridization temperatures.

The ASO method is especially useful in large-scale research applications, *e.g.*, screening for genetic diseases, various viruses or typing of HLA variants. With this assay, normal, carrier or mutant DNA sequences can be distinguished clearly. We obtained over ten-fold differences in normal/deletion-specific signal ratios among the three categories.

### Minisequencing Assay

The specific incorporation of labeled nucleotides with normal samples ( $^3\text{H}$ -dCTP) gave cpm values of 397–452, whereas  $^3\text{H}$ -dUTP gave only 25–49 cpm (Fig. 4). The ratios between these were 8- to 18-fold, giving straightforward results. Further, mutant samples were detected as clearly as the normal and mutant control DNAs. When carrier samples were tested, the signal levels were ~50% of those obtained with homozygous samples and the dCTP/dUTP ratios were between 1.2 and 1.8.

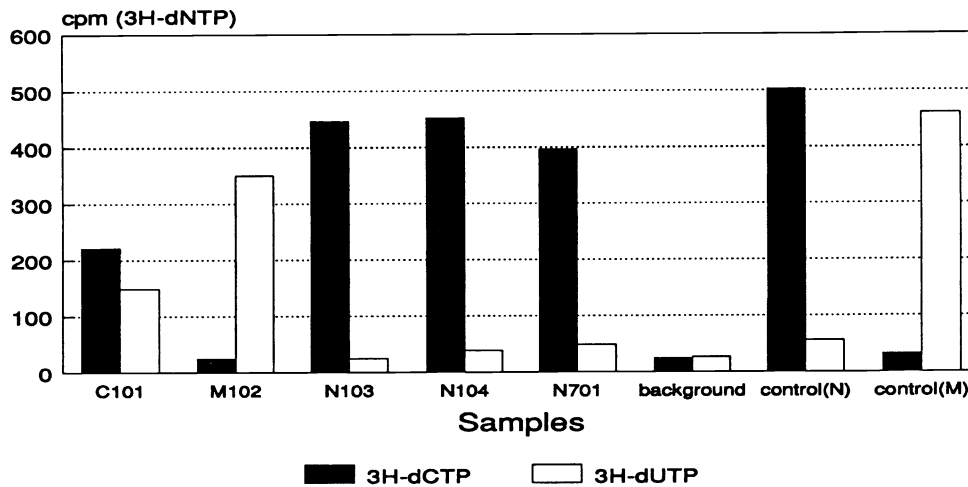


Fig. 4. Detection of  $\Delta\text{F508}$  mutation with  $^3\text{H}$ -labeled nucleotides and Microbeta™; ■ =  $^3\text{H}$ -dCTP; □ =  $^3\text{H}$ -dUTP

### CONCLUSION

With these assays, an amplified DNA sample could be interpreted in 3 h as normal, carrier or mutant for the  $\Delta\text{F508}$  mutation. Assays can be automated using the microtitration plate format and Microbeta™, allowing a higher sample throughput, saving both laboratory time and costs. Quantitative results can also be obtained using the Microbeta™ counter and the solution hybridization assay principle, resulting in a more precise follow-up of biological phenomena. A simple microtitration strip-based procedure facilitates automation and makes use of available laboratory equipment. We used a Microbeta™ 1450 with six detectors to enhance the throughput of this method. Our method offers a versatile, simple tool for detecting amplified sequences with labeled DNA probes.

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