A method of one-step enzyme labelling of short oligonucleotide probes for filter hybridisation

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ABSTRACT

Here we describe a method of labelling short oligonucleotide probes with enzyme without purification or chemical modifications. Biotinylated oligonucleotides as short as 10 nt are coupled with streptavidin-conjugated enzyme, hybridised and detected with enzyme-triggered chemiluminescence. The detection of hybridisation signal is linear for two orders of magnitude of target dilution. It is shown to be comparable in sensitivity with standard procedures and with radioactive detection. The method is quick, simple and has potential for automation of large-scale oligonucleotide hybridisation and multiplex sequencing.

Oligonucleotide probes are widely used for hybridisation with filter-bound DNA. Very short oligonucleotides, 10 nt or less, provide high specificity (1) and have a number of applications, for example fingerprinting of sequences by systematic oligonucleotide hybridisation (2–5).

Conventionally, detection is based on isotopic labelling of the probes. A non-radioactive method of detection could reduce the cost and time needed for the analysis. Unfortunately, most of the existing fluorophores have low signal to background ratio for nylon membranes, a typical support for DNA immobilisation. Enzyme amplification of hybridisation signals, a highly sensitive alternative technique, usually includes a three-step procedure preceding the development with chemiluminescent or fluorescent substrates and detection: (i) blocking of the membrane after hybridisation of a biotin- or digoxigenin-labelled probe; (ii) incubation with enzyme–streptavidin or enzyme–antibody conjugates; and (iii) washing off of conjugate (6). Although the methods for direct conjugation of enzyme with DNA probe have greatly simplified the technique (7), the labelling of very short DNA probes, e.g. 10 nt, is incompatible with existing protocols. It is difficult to perform reliable purification of short oligonucleotides during intermediate chemical modifications. Thus non-radioactive detection of very short probes still relies on a three-step protocol.

In the present study we describe coupling of biotinylated 10 nt oligonucleotides with alkaline phosphatase via a streptavidin–biotin link, and hybridisation of the modified probe with no further purification compared to earlier protocols (7,8). Any chemical modifications of DNA along with intermediate purification are avoided. Biotin is incorporated via synthesis at the 5′-end and serves as a uniform label for coupling of any commercially available streptavidin–enzyme conjugates. The described method is as sensitive as the radioactive detection method and we did not observe reduction in sensitivity of hybridisation compared to the three-step protocol.

For coupling of enzyme 30 pmol of 5′-biotinylated oligonucleotides were incubated with 1 U of Str-AP (Streptavidin-Alkaline Phosphatase conjugate; Boehringer Mannheim, Cat#1089161), in 10 µl of 20 mM Tris pH 8.0 for 10 min at room temperature. Then 2 µl of 300 µM D-biotin (Sigma) solution were added. The hybridisation was carried out for 5 h in SSARC buffer (4× SSC, pH 7.0 and 7% (v/v) sodium lauryl sarcosinate (‘Sarkosyl’ NL30, BDH)) at 6 µM probe concentration. All steps were carried out at 4°C. After hybridisation, membranes were washed for 30 min in SSARC buffer and rinsed in developing buffer (0.1 M diethanolamine, pH 9.0, 0.5 M NaCl and 1 mM MgCl2). The membranes were immersed in 0.25 mM solution of CDP-Star (Boehringer Mannheim) in developing buffer, briefly dried on Whatman, wrapped and photographed using Lumi-Imager LAS100 (Boehringer Mannheim) after 15 min of incubation at room temperature in the dark. The signals were evaluated using manufacturer’s software. Probe and substrate were stripped off the membrane by incubation in 0.1% SDS/0.5× SSC at 65°C.

The optimal molar ratio of streptavidin to biotin for probe coupling was ∼1.5:1. One unit of Str-AP was estimated as 50 pmol. The coupling procedure is very efficient for short oligonucleotides and essentially sequence independent. Although it is not clear how many streptavidin sites are available for biotin binding in a single Str-AP molecule, taking into account a non-cooperative character of biotin binding (9), we speculate that streptavidin binds a single biotinylated oligonucleotide rather than the theoretically possible four. We added excess of free biotin to the conjugated oligonucleotide prior to hybridisation to block free binding sites in streptavidin, which resulted in significantly reduced background. We expect that an exchange between oligonucleotide-linked biotin and free biotin is unlikely (9).

For comparison, a three-step protocol was performed as described by Maier et al. (10). After hybridisation and washing for 30 min in hybridisation buffer the membrane was incubated with the Str-AP conjugate (1 U in 5 ml) in hybridisation buffer for 15 min at 4°C, followed by two 10 min washings in SSARC buffer, and then processed as above prior to imaging.
Radioactive probes were prepared by phosphorylation of 30 pmol of non-biotinylated oligonucleotide with 50 µCi [γ-32P]ATP (2500 Ci/mmol, Amersham) in 10 µl reaction and hybridised at 6 nM probe concentration in SSARC buffer at 4°C. For 5 h. The filters were washed for 30 min at 4°C. Membranes were exposed to Fujifilm imaging plate for 12 h and scanned on a Fuji BAS 5000 system. Data analysis was performed using AIDA 2.0 software (Raytest).

To evaluate sensitivity and specificity of hybridisation we used four 15 nt oligonucleotides containing each of the four nucleotide substitutions in position 6 and corresponding complementary 5-biotinylated oligonucleotides, as outlined in Figure 1A, as model system. The four target oligonucleotides were transferred as a 3-fold dilution series to Hybond N+ membrane (Amersham) using vacuum dot-blotter. Membranes were treated according to the manufacturer’s protocol and pre-washed in SSARC buffer for 30 min at 65°C before the hybridisation. As revealed by blotting the radioactive target oligonucleotide membrane retained 1–2.5% of blotted oligonucleotide after washing and had saturation at 1 pmol/mm² (not shown). Nevertheless, the system of immobilised targets a few nucleotides longer than the probe reflects specificity of duplex formation better than a more complex target system, for which it might be difficult to discriminate the signal for the matching duplex over several mismatched ones.

We have compared hybridisation signals of the target dilution series detected by enzyme-coupled probe, by three-step procedure and by radioactivity. The sensitivity was essentially the same for all three methods achieved in 12 h of exposure for radioactivity and 15–30 min of exposure for chemiluminescence (Fig. 1B). Taking into account losses of target oligonucleotides of ~100-fold we estimate the minimum level of detection as <0.1 fmol/mm².

The dependence between the amount of DNA blotted and the hybridisation signal has also been tested. Enzyme-coupled probes were hybridised to six replicas of dilution series of target and the mean of signal values were plotted against target DNA amounts (Fig. 1C). The signal value is a function of the exposure time; therefore different CCD exposure times were examined. The highest signal to noise ratio was obtained for exposure time of 3–10 min. In independent experiments using two different 10 nt oligonucleotides, a linear response between the hybridisation signal has also been tested. Enzyme-coupled probes (not shown). Our results suggest that coupled proteins do not have significantly destabilise the interaction of short oligonucleotides with membrane-bound targets. The same was found for uncharged DNA analogues such as peptide nucleic acid (PNA) (not shown). The use of more stable PNA as hybridisation probes charged DNA analogues such as peptide nucleic acid (PNA) (not shown). The use of more stable PNA as hybridisation probes might circumvent the problem of low stability of oligonucleotides shorter than 10 nt.

The prospective of the direct probe labelling can be attractive for automated multiplex sequencing approach (6) and DNA typing methods using longer oligonucleotide probes. At ambient

Figure 2. Specificity of hybridisation of Str-AP probes with blotted dilution series of four oligonucleotide targets. Three filter replicas with 3-fold dilution series were used. The images were produced using X-ray film (Kodak), exposure time of 3 min. (A) Hybridisation of probe complementary to target C; (B) hybridisation with degenerate probe N complementary to each target was performed in order to examine whether all targets were blotted equally; and (C) hybridization of the probe complementary to target T.

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Sequence-specific hybridisation with enzyme coupled probes is shown in Figure 2. The values were measured on Lumi-Imager. The ratio of perfect to mismatch signals was at least 10-fold at higher amounts of spotted DNA, and increased dramatically with dilution, similar to the radioactive method.

The proposed labelling method can be extended for different commercially available streptavidin- or even anti-digoxigenin antibodies-conjugated reporter groups. It was possible to use Streptavidin–phycoerythrin conjugate (molecular weight 300 kDa) coupled to biotinylated oligonucleotides as hybridisation probes (not shown). Our results suggest that coupled proteins do not significantly destabilise the interaction of short oligonucleotides with membrane-bound targets. The same was found for uncharged DNA analogues such as peptide nucleic acid (PNA) (not shown). The use of more stable PNA as hybridisation probes might circumvent the problem of low stability of oligonucleotides shorter than 10 nt.

The prospective of the direct probe labelling can be attractive for automated multiplex sequencing approach (6) and DNA typing methods using longer oligonucleotide probes. At ambient
temperature, alkaline phosphatase retains activity during the hybridisation in the presence of 5% SDS. Str-AP-labelled probes are fully compatible with already existing automated protocols, e.g. direct blotting and imaging of membranes (13). The data obtained suggest that enzyme-coupled oligonucleotides can be a cost and time saving alternative to radioactive probes for a variety of applications.

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