Preparation of pure oligonucleotide-alkaline phosphatase conjugates

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The utility and sensitivity of enzyme-labeled oligonucleotide probes have been described (1, 2). However, most methods used for their preparation require the use of extensive column chromatography, polyacrylamide gel electrophoresis, or high-pressure liquid chromatography to yield a pure product (3-5). Commercial kits offer a simple alternative but typically 1) are expensive, 2) utilize freeze-dried enzyme, 3) rely on size exclusion chromatography, which may yield an impure product. Here we describe methods for the conjugation of 5'-amine and 5'-thiol-labeled oligonucleotides to alkaline phosphatase. These methods employ inexpensive consumables to produce pure conjugates.

Oligonucleotides were initially 5'-labeled during automated synthesis using 6-(4-monomethoxytritylaminohexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite and 1-O-Dimethoxytrityl-hexyl-disulfide, 1'-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (Glen Research, Sterling, VA) and purified by reverse phase column chromatography.

The procedure for the conjugation of 5'-amine-labeled oligonucleotides to alkaline phosphatase was as follows: To 15 nmol of 5'-amine-labeled oligonucleotide suspended in 12 μl 50 mM N-(3-sulfopropyl) morpholine (MOPS) pH 7.0, 4 μmol of ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester) (EGS; Sigma, St Louis, MO) in 28 μl dimethylformamide was added, and the mixture thoroughly vortexed. After incubating for 15 min at 21°C, 1 ml 100 mM NaCl, 50 mM MOPS pH 7.0 was added and the mixture applied to a pre-equilibrated Qiagen tip-20 (Qiagen, FRG). Excess EGS was washed from the column and the activated oligonucleotide selectively eluted according to the manufacturer's instructions for synthetic oligonucleotide purification. Dialfiltration with a pre-rinsed Centricon-3 device (Amicon, Beverly, MA) was used to transfer the activated oligonucleotide into 100 μl 3 M NaCl, 30 mM triethanolamine pH 7.6, 5 mM MgCl₂, 0.2 mM ZnCl₂ (AP-buffer). Calf intestinal alkaline phosphatase (Biozyme, San Diego, CA; 15 nmol in 100 μl AP-buffer) was combined with the activated oligonucleotide and incubated at 21°C for 4-16 h in the dark. The reaction mixture was suspended into 3 ml 50 mM MOPS pH 7.0 and applied to a pre-equilibrated Qiagen tip-20. Unreacted alkaline phosphatase was washed from the column, and the oligonucleotide-alkaline phosphatase conjugate and free oligonucleotide were eluted as described above. The eluate was placed into a Centricon-30 device, concentrated, and washed twice with 1 ml AP-buffer to remove free oligonucleotide. The final product, containing the purified conjugate, was stored in 1 ml AP-buffer at 4°C.

Thiol-labeled oligonucleotides were conjugated to alkaline phosphatase using the following procedure: To 20 nmol calf intestinal alkaline phosphatase suspended in 250 μl 50 mM MOPS pH 7.5, 1.5 μmol sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC; Pierce, Rockford, IL) was added, and the mixture gently vortexed. After incubating for 45 min at 21°C, 1.75 ml 50 mM MOPS pH 7.0 was added. The diluted mixture was placed into a pre-rinsed Centricon-30 device, concentrated, and washed twice with 1 ml 50 mM MOPS pH 7.0 to remove excess sulfo-SMCC. The activated alkaline phosphatase was combined with 15 nmol 5'-thiol-labeled oligonucleotide, suspended into 100 μl 50 mM MOPS pH 7.0, 2.5 mM EDTA and incubated at 21°C for 4 h in the dark. Oligonucleotide-alkaline phosphatase conjugate was separated from free enzyme and oligonucleotide as described above.

The conjugates were characterized using a protein assay kit (Bio-Rad, Richmond, CA), and by observing the relative fluorescence enhancement subsequent to acridine orange (AO; Sigma, St Louis, MO) binding to DNA (1.5 μM AO in 10 mM sodium phosphate pH 7.0, Ex 462 nm, Em 523 nm). Conjugates composed of 1-2 oligonucleotides were generated using both procedures. The yields with respect to alkaline phosphatase were 15% ± 10% using 5'-amine-labeled oligonucleotides and 69% ± 15% for thiol-modified oligonucleotides. The molar absorptivity (ε = 1.4 × 10⁶ at A₂₈₀) of calf intestinal alkaline phosphatase (6) can be used to estimate the product yield, however this value will be overstated 20-40%. Because the optimal probe concentration is in the range of 0.25-1 nM (1), either method should yield sufficient oligonucleotide-alkaline phosphatase conjugate for most purposes.

The functionality of our probes was assessed by comparison to probes of the identical sequence prepared using a commercial kit (Lightsmith 1; Promega, Madison, WI) and as described by Jablonski et al. (3). Southern blots of pBLSV13 DNA (an 11.4 kbp plasmid containing a single copy of the target sequence) (7) on Biodyne-B nylon membranes (Boll Biosupport, Glen Cove, NY) were prepared as previously described (8). Membranes were prehybridized in 3% w/v Hammarsten casein (Gallard-Schlesenger, Carle Place, NY), 5 x SSC (1 x SSC = 150 mM NaCl, 15 mM Na₃C₂H₄O₇; pH 7.0) at 49°C for 60 min. Hybridization was performed in 10% w/v PEG 8000, 0.4 mg/ml heparin sulfate, 25 mM SDS, 5 x SSC and 0.3 nM probe at 49°C.
Figure 1. Southern analysis of oligonucleotide-alkaline phosphatase probe functionality. Four identical membranes containing linearized pBLV913 DNA were hybridized with a 25 nt alkaline phosphatase labeled probe prepared from a 5'-amine (A) or 5'-thiol (B) using our methods, 5'-amine using the procedure of Jablonski (C) and a 5'-amine labeled oligonucleotide using the Lightsmith kit (D). Attomoles of target DNA are indicated above the membranes. The incubation and exposure times were 16 and 2 h, respectively.

for 45 min. After hybridization, the membranes were washed three times in 35 mM SDS, 1 x SSC at 49°C for 5 min, twice in 1 x SSC at ambient temperature for 5 min and once in 0.5 M NaHCO₃-Na₂CO₃ pH 9.5, 1 mM MgCl₂ for 2 min at ambient temperature. Visualization with Lumi-Phos 530 (Boehringer Mannheim, Indianapolis, IN) was as described by the manufacturer. The similarity of band intensities in exposures A, B and C (Figure 1) demonstrate that our probes are of equivalent sensitivity to probes prepared using a previously published method (3). This result is not surprising since the same quality of alkaline phosphatase was used to prepare conjugates of a similar oligonucleotide:enzyme ratio in these methods. As such, the decreased relative sensitivity of the Lightsmith conjugated probe may reflect a lower enzyme specific activity.

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