5532–5533 Nucleic Acids Research, 1993, Vol. 21, No. 23

Preparation of pure oligonucleotide-alkaline phosphatase conjugates

R.A.Reyes and G.L.Cockerell
Department of Pathology, Colorado State University College of Veterinary Medicine and Biomedical Sciences, Fort Collins, CO 80523, USA

Received June 7, 1993; Revised and Accepted October 6, 1993

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-monomethoxytritylamino)hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite or 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 (Diagen, FRG). Excess EGS was washed from the column and the activated oligonucleotide selectively eluted according to the manufacturer's instructions for synthetic oligonucleotide purification. Diafiltration 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 $MgCl_2$, 0.2 mM $ZnCl_2$ (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 sulfosuccinimidyl 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 ($\epsilon = 1.4\times10^5$ at A_{280}) 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 pBLV913 DNA (an 11.4 kbp plasmid containing a single copy of the target sequence) (7) on Biodyne-B nylon membranes (Pall 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× SSC (1× SSC = 150 mM NaCl, 15 mM $Na_3C_6H_5O_7$; 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× SSC and 0.3 nM probe at 49°C

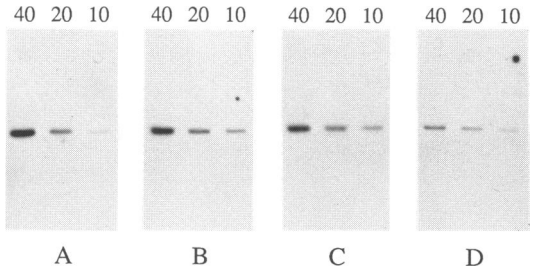

Figure 1. Southern analysis of oligonucleotide-alkaline phosphatase probe functionality. Four identical membranes containing linearized pBLV913 DNA were hybridized with a 25 nt alkaline phasphatase labeled probe prepared from a 5′-amine (**A**) or 5′-thiol (**B**) using our methods, 5′-amine using the procedure of Jablonski (**C**) and a 5′-amine labeled oligonucleotide using the Lightsmith I 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× SSC at 49°C for 5 min, twice in 1× SSC at ambient temperature for 5 min and once in 0.5 M $NaHCO_3$-Na_2CO_3 pH 9.5, 1 mM $MgCl_2$ 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.

ACKNOWLEDGEMENT

This work was supported in part by a National Research Service Award (5-F31 GM14859-02) from the National Institute of General Medical Sciences.

REFERENCES

1. Cate,R.L., Ehrenfels,C.W., Wysk,M., Tzard,R., Voyta,J.C., Murphy,O.J. and Bronstein,I. (1991) *GATA* **8**, 102–106.
2. Pollard-Knight,D. (1990) *Technique* **3**, 113–132.
3. Jablonski,E., Moomaw,E.W., Tullis,R.H. and Ruth,J.L. (1986) *Nucleic Acids Res.* **14**, 6115–6128.
4. Urdea,M.S., Warner,B.D., Running,J.A., Stempein,M., Clyne,J. and Hom,T. (1988) *Nucleic Acids Res.* **16**, 4937–4955.
5. Farmar,J.G. and Castaneda,M. (1991) *Biotechniques* **11**, 588–589.
6. McComb,R.B., Bowers,G.N. and Posen,S. (1979) *Alkaline Phosphatase*. Plenum Press, New York.
7. Rovnak,J., Boyd,A.L., Casey,J.W., Gonda,M.A., Jensen,W.A. and Cockerell,G.L. (1993) *J. Virol.* in press.
8. Dubitsky,A., Brown,J. and Brandwein,H. (1992) *Biotechniques* **13**, 392–400.