Benchmarks

Efficient 5′-End Labeling of Oligonucleotides Containing Self-Complementary Sequences

Synthetic oligonucleotides have become basic tools in molecular biology research and are commonly used for cloning and characterization of genes, analysis of gene expression and so on. Frequently, the success of techniques such as DNA and RNA sequencing, screening of libraries, nuclease S1 and RNase protection assays or polymerase chain reaction (PCR) amplification depends on appropriate design and labeling of the oligonucleotides used. At the time of oligonucleotide design, sequences with the potential to self-anneal should be avoided because self-complementarity of oligonucleotides is a factor that directly interferes with sequencing, hybridization and labeling processes. However, when oligonucleotides are designed for cloning purposes, they often include endonuclease restriction sites (e.g., palindromic sequences) to simplify the cloning process.

We report a method that allows the labeling of oligonucleotides even though they contain strong secondary structures. In the development of the technique, we took advantage of previous reports indicating that dimethyl sulfoxide (DMSO) reduces inter- and intra-strand annealing (4) and improves sequencing and PCRs (5). It has been shown that DMSO has its primary effect on melting temperature of primers (1). Thus, we assayed the effect of this denaturing agent on the labeling efficiency of oligonucleotides with the potential to form stable secondary structures. The oligonucleotide 70 II, shown in Figure 1, was chosen because of repeated failures to label it using the standard T4 polynucleotide kinase method (3). Prior to labeling, the oligonucleotide was incubated for 2 min at 70°C in the presence of 5% DMSO. Immediately, the tube was chilled on ice, and 1 µL (25 ng) of the oligonucleotide solution was added to the labeling reaction mixture (50 mM Tris-HCl, 10 mM MgCl$_2$, 0.1 mM EDTA, 5 mM dithiothreitol, 0.1 mM spermidine, pH 8.2), containing 50 µCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham International plc, Bucks, England, UK) and 10 U of T4 polynucleotide kinase (Boehringer Mannheim GmbH, Mannheim, Germany) in a final volume of 10 µL. The mixture was incubated at 37°C for 30 min. Afterwards, the reaction was stopped by the addition of 5 µL of 500 mM EDTA (pH 8.0), and the volume was adjusted to 100 µL with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The labeled oligonucleotide was purified using a Sephadex® G-50 Column (Pharmacia Biotech, Piscataway, NJ, USA), and the incorporated cpm were measured in a scintillation counter. In parallel, as a control, the same oligonucleotide was labeled using the standard technique without the DMSO treatment (3). Also, according to a suggestion from Oligos Etc. (Wilsonville, OR, USA), a labeling reaction was performed in the presence of 2× T4 polynucleotide kinase buffer (Boehringer Mannheim GmbH). This latter procedure was found to solve the labeling problems of some, but not all, oligonucleotides (unpublished). Table 1 summarizes the labeling efficiency values obtained by the different methods. The results indicate that denaturation of oligonucleotide 70 II in the presence of DMSO increased 20-fold the ^{32}P incorporation efficiency relative to standard methods. The high specific activity of the labeled, DMSO-treated oligonucleotide, 8.4×10^8 cpm/µg, should be noted.

The efficient labeling of oligonu-

Table 1. Incorporation Efficiency of ^{32}P into Different Oligonucleotides

Oligonucleotide	DMSO Pretreatment	Kinase Buffer Strength	cpm Incorporated*	Specific Activity (cpm/µg)
70 II	+	1×	$2.1 \pm 0.1 \times 10^7$	8.4×10^8
70 II	-	1×	$0.9 \pm 0.8 \times 10^6$	3.6×10^7
70 II	-	2×	$1.2 \pm 0.6 \times 10^6$	4.8×10^7
PE1	+	1×	$2.1 \pm 0.4 \times 10^7$	8.4×10^8
PE1	-	1×	$1.9 \pm 0.3 \times 10^7$	7.6×10^8
M13	+	1×	$1.5 \pm 0.3 \times 10^7$	6.0×10^8
M13	-	1×	$1.4 \pm 0.3 \times 10^7$	5.6×10^8

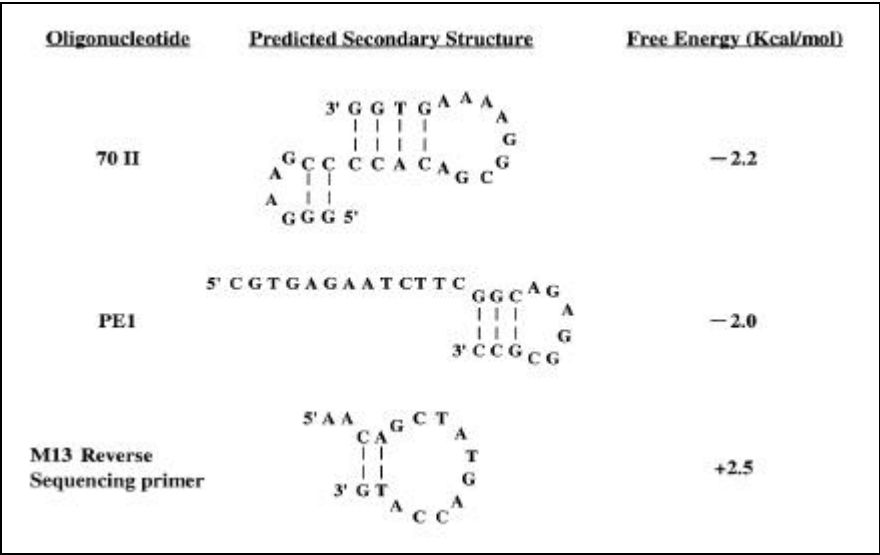*Mean values of three independent reactions.

Figure 1. Predicted secondary structures of the assayed oligonucleotides. These secondary structures were obtained using the University of Wisconsin Genetics Computer Group Fold Program (2).

cleotide 70 II in the presence of DMSO might be due to an opening of the secondary structure, involving the 5′ end of the oligonucleotide (Figure 1). To assess this possibility, the labeling efficiency, again in the presence and absence of DMSO, was assayed for an oligonucleotide that contains one self-annealing region that does not involve the 5′ end of the oligonucleotide (PE1; Figure 1). As shown in Table 1, this oligonucleotide was labeled with similar efficiencies in the absence or presence of DMSO, supporting, therefore, the above indicated mechanism for DMSO. In the present study, the labeling efficiencies in the presence and absence of DMSO of a widely used oligonucleotide, such as the M13 Reverse Sequencing Primer (Catalog No. 1201; New England Biolabs, Beverly, MA, USA), were also assayed to further demonstrate that the presence of DMSO has no inhibitory effects on la-

beling efficiency. In fact, although not statistically significant, the labeling efficiencies of oligonucleotides PE1 and M13 Reverse were slightly higher than those obtained in the absence of DMSO (Table 1). In summary, this protocol assures the efficient 5′-end labeling of oligonucleotides with self-annealing sequences without involving an increase in technical complexity on the previous one. Also, because DMSO has no inhibitory effects on 5′-end labeling of oligonucleotides by T4 polynucleotide kinase, we recommend the routine use of this method for 5′-end labeling of any oligonucleotide.

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Detection of Major Histocompatibility Complex Class I Antigens on the Surface of a Single Murine Blastocyst by Immuno-PCR

There have been several recent advances in the field of low-level antigen detection, including the development of stronger fluorochromes and chemiluminescent substrates for use in enzyme-linked immunosorbent assays (ELISAs), immunofluorescence-based staining and immunoblotting. Although these techniques can be useful, sometimes even greater sensitivity and specificity are required, particularly when working with limited amounts of sample material or when antigen density is very low.

One recently developed antigen detection technique that is extremely sensitive and reproducible is immuno-polymerase chain reaction (I-PCR), which combines the specificity of antigen-antibody interactions with the sensitivity of the PCR (5). In one variation of this technique, a bispecific protein chimera was engineered containing a protein A moiety (to bind to the Fc portion of any antibody) and a streptavidin moiety (to

bind to any biotinylated DNA molecule) (4). This biotinylated DNA molecule can be amplified by standard PCR techniques, and the product can be visualized by running on an electrophoresis gel and staining with ethidium bromide. The presence of a band of the appropriate molecular weight implies that the primary antibody bound to the appropriate antigen and the biotinylated DNA-chimera complex bound to the primary antibody. Using this chimeric protein, an approximate 10^5-fold increase in sensitivity over a colorimetric ELISA was obtained, and as few as 580 molecules of bovine serum albumin (BSA), which had been coated on the wells of a 96-well plate, were detected (5). To our knowledge, no one has tried I-PCR on actual tissue or cell samples using this chimeric protein.

We have developed a protocol that uses this bispecific chimera to detect murine major histocompatibility complex (MHC) class I molecules on the surface of preimplantation embryos. Unlike previous antigen detection methodologies, our protocol allows for cell surface protein detection on a single blastocyst. Previously, we established a conventional colorimetric ELISA procedure for the detection of cell surface proteins on blastocysts (1), but this was found to require at least 10–15 embryos to generate a signal above background levels. We wanted to develop a simple and easy, yet sensitive and reliable, technique that could detect the expression of MHC class I molecules on the surface of single embryos collected at the blastocyst stage and also in embryos collected at earlier stages and cultured in vitro. In addition, this method would have other potential applications, including the analysis of antigens in genetic segregation studies, the evaluation of microinjection efficiency and the investigation of changes in protein expression patterns (i.e., in response to various drug or hormonal treatments).

Host bacteria lysogen BL21(DE3) pLysS (Novagen, Madison, WI, USA) were transformed with the plasmid pTSAPA-2 as described (4). Expression cloning and subsequent purification of the recombinant protein were performed according to the protocol of Sano and Cantor (4). The purified chi-

mera was stored at -20°C in 10-μL aliquots.

A 2.67-kb *Hin*dIII-*Acc*I fragment from the plasmid pUC19 (Stratagene, La Jolla, CA, USA) was biotinylated using the Klenow fragment of DNA polymerase (Amersham, Arlington Heights, IL, USA) to incorporate biotinylated dATP (Life Technologies, Gaithersburg, MD, USA) in the creation of a blunt-ended molecule of DNA according to the manufacturer's protocol. The DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) at the desired volume. The biotinylated pUC19 fragment and purified recombinant chimera were then mixed at a molar ratio of 4:1 (biotin:biotin-binding site), and the resulting chimera-DNA complex was refrigerated at 4°C. A stock solution of 1×10^{-11} M was diluted in the same buffer used to collect blastocyst-stage embryos.

For detection of MHC class I proteins on the surface of a single blastocyst, embryos were collected from C57BL/6 mice into phosphate-buffered saline (PBS) containing 1% BSA and 0.1% NaN_3 (PBSAZ) into glass depression slides (watch-glasses). Blastocysts that were morphologically normal were pooled and washed once in 500 μL of PBSAZ. We found that it is essential that the zona pellucida be completely removed from the embryos to eliminate background signals. This was done by incubating the embryos in 200 μL of acidic Tyrode's solution (pH 2.5) for 1–2 min and then washing three times in PBSAZ. First antibody, an anti-class I MHC monoclonal antibody (anti-D^b), was diluted in PBSAZ at 50–500 μg/mL, and embryos were placed into a watch-glass containing 100–200 μL of the antibody. It should be noted that the antibody must be protein A-purified, because assays performed with ascites tended to yield too much background. The control was either PBSAZ only or a nonspecific first antibody to sheep red blood cells (TIB109). Embryos were incubated at 4°C for 1–2 h and washed 5 times in PBSAZ + 0.02% Tween® 20 (PBST), followed by 5 additional washes in PBSAZ. The washed embryos were then incubated in 1×10^{-15} or 2×10^{-13} M of chimera-biotinylated DNA complex at 4°C for 45 min to 1 h. Embryos were washed as above, and