Immobilized metal affinity chromatography of DNA

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ABSTRACT

Many of the most widely employed operations in molecular biology hinge upon the use of single-stranded DNA as a probe or template. Here we report a straightforward method by which to produce long single-stranded DNA molecules using the polymerase chain reaction (PCR) in combination with immobilized metal affinity chromatography (IMAC). We demonstrate that a tag consisting of six successive 6-histaminylpurine (H) residues (H₆-tag) endows a DNA strand with selective retentivity onto a Ni²⁺-NTA-agarose chromatography matrix. The H₆-tagged strand can then be eluted from the resin using 200 mM imidazole. Quantitative phosphor-imaging analysis revealed that the PCR/IMAC procedure typically yields unmodified strands comprising >95% of the bound fractions. DNA strands generated in this manner are shown to be excellent substrates for template-directed polymerization. The chemistry reported herein should facilitate a wide variety of operations in molecular biology, including automated DNA sequencing, hybridization screening of DNA libraries, assembly of gene cassettes, run-off transcription, site-directed mutagenesis and footprinting of protein–DNA complexes by template-directed interference footprinting.

INTRODUCTION

Many of the most widely employed operations in molecular biology hinge upon the use of single-stranded DNA as a probe or template (1). Whereas single strands of DNA containing up to ~100 residues can readily be produced by solid-phase synthesis, longer oligodeoxynucleotides must be generated through enzymatic methods such as the polymerase chain reaction (PCR) (2–4). Biochemical procedures for the synthesis of mixed-sequence DNA yield double-stranded products. One exception is the so-called asymmetric PCR procedure (3–8) in which one PCR primer is used in large excess over the other. Under these conditions, the exponential phase of PCR amplification proceeds until the supply of limiting primer is exhausted by the production of duplex DNA; after this, the primer present in excess supports linear amplification of only the strand emanating from it. We and others have used asymmetric PCR with success; however, in our hands (6–8) the method produces highly variable yields of single-stranded product, even in parallel reactions aliquoted from the same master reaction mixture. The yields of duplex DNA obtained via asymmetric PCR reactions, on the other hand, are less variable. An affinity-based procedure that employs the binding of biotin-labeled oligonucleotides to streptavidin-linked beads has also been employed in strand separation (9,10), but invariably one strand is lost to the beads and the procedure is incompatible with strongly denaturing conditions. Thus there exists the need for a truly general method by which to resolve double-stranded PCR products into its constituent strands. Such resolution is rendered difficult by the similarities in macroscopic physical properties such as size and charge of the two complementary strands, and by the requirement that it be carried out in the presence of strong denaturants such as urea or guanidinium hydrochloride. Here we report the development of a highly effective, operationally straightforward method for resolving duplex DNA into its constituent strands, using immobilized metal affinity chromatography (IMAC; 11).

MATERIALS AND METHODS

Synthesis and purification of H₆-tagged oligonucleotides

The H₆-tagged oligonucleotides were synthesized by the convertible nucleoside approach (12–15) using the O’-phenyl-2’-deoxyinosine (dI) phosphoramidite (16,17) along with PAC phosphoramidites (Pharmacia). The resin-bound oligonucleotide 5’-d(G[φO₆]-AGCG-GATAACATTTTCACACAGG] and 5’-d(G[φO₆]TCGTGACTG-GGAAAACCTGGCG]) were deprotected by treatment with 1 ml concentrated (14 M) aqueous ammonium hydroxide at room temperature for 4 h and lyophilized to dryness on a Speed Vac (Savant). The DNA pellets were redissolved in 100 µl 5 M aqueous histidine and incubated at 55°C for 14 h. After cooling to room temperature, 300 µl absolute ethanol (kept at ~20°C) was added, the mixture was chilled on crushed solid CO₂ for 30 min, then centrifuged at 16 000 g for 30 min. The supernatants were discarded and the pellets washed with 200 ml 80% (v/v) aqueous ethanol solution (~20°C). The pellets were dried on a Speed Vac, redissolved in 200 µl formamide loading buffer (95% aqueous formamide, 20 mM EDTA, 0.05% each bromophenol blue and xylene cyanol), heated to 90°C for 5 min, then loaded onto a 20% (19:1 acrylamide:bis) polyacrylamide gel (20 x 20 cm) containing 7 M urea. The gels were pre-run at 300–500 V in TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8) for at least 1 h prior to loading the DNA. Following electrophoresis, the gel was removed from the glass plates, enclosed in Saran Wrap and placed over a TLC plate impregnated with fluorescent dye. The full-length DNA band was visualized using a hand-held UV

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lamp, excised from the gel with a sharp razor blade, placed in a 50 ml Falcon tube and crushed thoroughly using the polished end of a glass stirring rod. The crushed gel was soaked overnight at 37°C in 10 m 1 M triethylammonium bicarbonate (TEAB), pH 8.0. The supernatant was transferred to a new Falcon tube, and the crushed polycrylamide was further extracted once with 5 ml M TEAB. The combined TEAB solutions were loaded onto a C18 Sep-Pak cartridge (Waters), which had bee pre-washed by successive throughput of 5 ml 100% CH3CN and 15 ml 50 mM TEAB. Following loading of the DNA, the Sep-Pak was washed with 2 ml 5% CH3CN/95% 50 mM TEAB and eluted with 10 ml 30% CH3CN/70% 50 mM TEAB into 1.5 ml Eppendorf tubes. The fractions were assayed by UV spectrophotometry, and those that contained a significant A260 were lyophilized to dryness in a SpeedVac. The lyophilized DNA pellets was combined in 50µl TE buffer to obtain an oligonucleotide stock solution that was used directly in subsequent experiments. This procedure typically yields ∼150 nmol highly purified H6-tagged oligonucleotide using a 200 nmol resin; comparable yields were obtained for untagged oligonucleotides purified by the same procedure. We have not investigated purification of the crude H6-tagged oligonucleotides using Ni2+-NTA chromatography, but this should work and would be sufficient for most applications.

**Nucleoside composition analysis**

The oligonucleotide sample (3 nmol) was digested with 0.2 U snake venom phosphodiesterase (Pharmacia) and 50 U Serratio endonuclease (‘Benzonase’, Merck) in 50 µl buffer containing 100 mM NaCl, 14 mM MgCl2, 100 mM Tris-HCl, pH 9.0 at room temperature for 2 h, then at 37°C for 2 h. The buffer was adjusted to 100 µl 0.1 mM ZnCl2, 50 mM NaCl, 17 mM MgCl2, 200 mM, 10 mM β-mercaptoethanol, 200 mM Tris-HCl, pH 9.0, and 1 U calf intestinal alkaline phosphatase (Boehringer-Mannheim) was added, and the resulting mixture was incubated at 42°C for 2 h. The digestion mixture was clarified by passage through a Millex Lp filter and analyzed by reversed-phase HPLC (Beckman Ultrasphere ODS, 4.6 × 250 mm) employing a photo diode array detector (Hewlett Packard LC 1090; Solvent A: 0.02 M KH2PO4, pH 5.6; Solvent B: 60:40 CH3OH/H2O: 1.5 mM/min; elution program: isocratic A for one min, 0–25% B in 10 min, 25–100% in 5 min, isocratic B for 10 min). Nucleosides were identified by comparison with authentic standards. A mock digest containing all digestion components except the oligonucleotide carried out as a control for contaminants introduced along with the buffers and enzymes.

**Procedure for IMAC resolution of PCR-amplified DNA**

PCR reactions, which follow standard procedures (4), employ 2.5 U Taq polymerase (Gibco/BRL), 50 pmol each primer and 0.2–0.5 µg supercoiled plasmid template in a reaction volume of 100 µl. To remove excess PCR reagents, 1 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8) is added to the crude reaction mixture, which is then concentrated to a total volume of 50 µl using a centrifugal dialysis cartridge (Centricon 30, Amicon). The DNA solution is transferred to an Eppendorf tube, to which is added 150 µl binding buffer (6 M guanidine-HCl, 10 mM Tris–HCl, pH 8.2). This mixture is heated for 5 min at 90°C. Separately, to a 1.5 ml Eppendorf tube is added 250 µl (bed volume) Ni2+-NTA-agarose resin and 1 ml binding buffer at room temperature. The hot DNA solution is added to the suspension of resin, and the resulting mixture is mixed for 1–1.5 min by vigorous shaking or repeated pipetting (longer mixing times result in reduced yields). The mixture is transferred to an empty 5 ml fritted column (Qiagen), and the flow-through (‘unbound’ fraction), which contains the unmodified strand, is collected into an Eppendorf tube. After repeated pipetting to ensure complete mixing, the unbound fractions are aliquoted into four Eppendorf tubes (300 µl each) and held aside for further processing. Next, the resin is washed with 1 ml washing buffer (10 mM Tris–HCl, 5 mM imidazole, pH 8.0), which is discarded. The H6-containing strand is then eluted in 1.2 ml 200 mM aqueous imidazole solution. The imidazole eluate (‘bound’ fraction) is mixed thoroughly and aliquoted into four Eppendorf tubes (300 µl each), to each of which is added 30 µl 100 mM ethanolic 1,10-phenanthroline and 30 µl 3 M aqueous NaOAc. No additional salt is added to the unbound fractions. To each of the unbound and bound fractions is added 900 µl absolute ethanol (stored at −20°C), then the tubes are vortexed briefly and chilled for 30 min on dry powdered CO2. The tubes are microcentrifuged for 30 min at 16,000 g. The supernatant is removed and the pellet washed with 200 µl 80% aqueous EtOH (−20°C). Following removal of the ethanol solution, the tubes are dried by centrifugal lyophilization (SpeedVac, Savant). To each dry tube was added 50 µl TE buffer. The DNA concentration was determined by UV spectrophotometry.

**Dideoxy (Sanger) sequencing reactions**

Sanger sequencing employed the Sequenase kit version 2.0 (US Biochemicals) using the protocol supplied by the manufacturer for 35S sequencing.

**RESULTS AND DISCUSSION**

Employed widely in affinity purification of proteins, IMAC relies on the ability of a strongly metal-coordinating polypeptide sequence (18) such as hexa-histidine (His6) (19,20) to bind specifically to a chromatography matrix containing a tightly chelated metal ion, usually Ni2+ (21–23). Importantly, IMAC can

![Figure 1](image)

**Figure 1.** Synthesis of H6-tagged oligonucleotides by the convertible nucleoside approach. Treatment of an oligonucleotide bearing the convertible base O6-phenylinosine (Ø) with aqueous histamine results in displacement of the O6-phenyl group to furnish 6-histaminylpurine (H). The H6-tag consists of six successive 2′-deoxyribo-H nucleotide (dH) residues (box).
by mild ammonia treatment (conc. NH₄OH, rt, 4 h), lyophilized φ
bound the resin, >95% of that from the H₆-tagged oligonucleo-
2a
oligonucleotides (Ni²⁺-nitrilotriacetic acid (NTA)-agarose resin, commercially
3808
nucleoside composition analysis of the H₆-tagged oligonucleotide
and then analyzed for constituent nucleosides by enzymatic
digestion and HPLC. Figure 2 shows the HPLC trace for the
products from enzymatic digestion of 1a with snake venom phosphodiesterase, benzonase and alkaline phosphatase. X is a
contaminant peak that is introduced along with snake venom phosphodiesterase.
The UV spectrum of X does not match that of any ordinary ²-deoxyribonucleoside.
The retention times and UV spectra of the four native nucleosides and the
modified nucleoside (dH) were identical to those of authentic standards (not
shown). The authentic standard for dH was generated by histamine displacement
on O₆-phenyl-2′-deoxynosine (15).

be carried out in the presence of urea and guanidinium hydrochloride, because these additives do not interfere with the specific metal–ligand interactions on which the technique is
based. In principle, this insensitivity to denaturants makes IMAC uniquely suited to the problem of DNA strand resolution. Implementation of an IMAC-based DNA purification strategy
required the development of a polynucleotide equivalent of His₆.
Analogous to His₆. As an initial test of this concept, we
synthesized DNA sequencing primers containing six successive
6-histaminylpurine (H) residues (H₆-tag) added onto the 5′
ends of the dH is evident. The peak denoted X is a contaminant
peak from the native nucleosides (dC, dG, T, dA), a peak due to the histamine-modified nucleoside dH is evident. This peak denoted X is a contaminant present in the mock digest, having been introduced along with the
snake venom phosphodiesterase. Importantly, a peak at the retention
time of dH was not observed, thus indicating complete conversion
of dH to dH. To assess the ability of the H₆-tag to endow DNA with
selective reactivity on a Ni²⁺-charged chelate resin, the H₆-tagged oligonucleotides (2a/2b) and their unmodified counterparts (1b/2b)
were 5′-end labeled with ³²P and then passed in parallel through a Ni²⁺-nitrilotriacetic acid (NTA)-agarose resin, commercially
available from Qiagen (Chatsworth, CA) (17,18). Whereas <5% of the radioactivity derived from the unmodified oligonucleotides
bound the resin, >95% of that from the H₆-tagged oligonucleo-
tides was adsorbed by the resin. All but a trace amount of the
bound radioactivity could be eluted from the resin upon washing
with 200 mM aqueous imidazole. These data demonstrate that
H₆-tagged oligonucleotides can be selectively retained on
Ni²⁺-NTA agarose, and efficiently eluted from the matrix by
aqueous imidazole.

For the present IMAC-based strategy to be of significant practical value, it must be capable of resolving PCR-amplified duplex DNA into its two component strands. To test this application directly, we used one H₆-tagged primer plus one
unmodified primer to amplify a 183 base-pair segment of the
plasmid pUC18-mARRE2 (24; Fig. 3a and b). pUC18-mARRE2
was derived from the commercial cloning vector pUC18 by inserting a segment of the murine interleukin-2 enhancer into the
BamH I site. We chose a pUC18 derivative for these experiments
because such plasmids are widely employed in cloning and other
subsequent manipulation that involve single-stranded DNA.
Primers 1b and 2b, commercially available oligonucleotides that
are commonly used to sequence pUC18 derivatives, were used in
combination with the corresponding H₆-tagged oligonucleotides
1a and 2a to generate PCR products having H₆-tagged on any one
strand. Namely, the PCR product generated using primers 1a + 2b
will bear the H₆-tag on the ‘top’ strand only, whereas that
generated using 2a + 1b will bear the H₆-tag on the ‘bottom’ strand
only (the designation of ‘top’ and ‘bottom’ strands refers to the
sequence as illustrated in Figure 3a). The yield of PCR product
formed in these reactions was no less than that observed in parallel
reactions with only unmodified primers (data not shown), thus
indicating that the H₆-tag does not affect PCR amplification
adversely. The PCR products were denatured in 6 M guanidi-
nium-HCl, then incubated batchwise with Ni²⁺-NTA-agarose.
Following removal of the supernatant, which contains unbound
DNA, the resin was washed and the bound DNA eluted with
200 mM imidazole. Aliquots of the crude PCR product and the
two DNA-containing fractions from the IMAC step were 5′-end
labeled with ³²P and analyzed by denaturing polyacrylamide gel
electrophoresis (PAGE). The use of PAGE as an assay for strand
resolution was made possible by the slightly reduced mobility of
H₆-tagged DNA strands relative to their complementary strands
generated by PCR (Fig. 4, lanes 1 and 3). The retarded mobility
of the H₆-tagged strand relative to the unmodified strand may
arise from the partial positive charge of its six pendant imidazole
moieties at pH 8 and a greater length, due to incomplete
polymerization of the T₆-stretch opposite H₆ during PCR. The
identities of the strands were independently verified by PAGE
analysis of PCR reactions that employed one ³²P-labeled primer
and one non-radioactive primer. Although this slight difference
in mobility provides a convenient means of assaying the strand
resolution by IMAC, it is not sufficiently large to permit
preparative resolution of relatively long PCR products by gel
electrophoresis and extraction. The slight difference in intensity of
the two bands in lanes 1 and 4 of Figure 4 results from
differential kinetics of end-labeling, rather than differences in
quantity of the DNA. The unbound fractions from IMAC (lanes
2 and 5) contained primarily the faster-migrating species, which
corresponds to the respective unmodified strands, whereas the
imidazole-eluted bound fractions (lanes 3 and 6) contained
primarily the respective H₆-tagged strands. Quantitative phos-
horimaging analysis from repeated runs of the strand resolution
procedure revealed that the unmodified strand typically comprise
>90% of the unbound DNA and the H₆-tagged DNA comprise
>95% of the bound fractions. Thus we conclude that IMAC

Figure 2. HPLC analysis of the products from enzymatic digestion of 1a with
snake venom phosphodiesterase, benzonase and alkaline phosphatase. X is a
contaminant peak that is introduced along with snake venom phosphodiesterase.

![HPLC trace](image-url)
Figure 3. Overview of procedure for production of long single-stranded DNA molecules using the polymerase chain reaction (PCR) followed by immobilized metal affinity chromatography (IMAC). (a) Primers and double-stranded PCR template used in this study. Primers 1a/1b contain a sequence that is identical to a stretch (shaded) of the ‘top’ strand of the plasmid pUC18-mARRE2; they are extended in the rightward direction (5′→3′) during PCR. Primers 2a/2b contain a sequence that is identical to a stretch (shaded) of the ‘bottom’ strand of pUC18-mARRE2; they are extended in the leftward direction. The shaded sequence in primers 1a and 2a denotes the H_{6}-tag. E and H denote the EcoRI and HindIII sites, respectively, of the pUC18 polylinker; a stretch of the murine IL-2 enhancer (mARRE2, hatched) was inserted into the BamHI site to generate pUC18-mARRE2. (b) Schematic illustration of the procedure for PCR amplification to produce duplex DNA containing a H_{6}-tag on one strand, and resolution of the two constituent strands using IMAC.

cleanly resolves a singly H_{6}-tagged PCR product into its two component strands.

To assay the biological activity of single-stranded DNA generated through the PCR-IMAC sequence, we employed the resolved strands as templates in Sanger dideoxy sequencing runs. Initially, the unmodified strands were found to be excellent sequencing templates, but the H_{6}-tagged strands failed to support template-directed polymerization. Eventually, it was discovered that the H_{6}-tagged DNA solutions contain adventitious Ni^{2+} in amounts sufficient to cause profound inhibition of the DNA polymerase enzyme. This problem was overcome simply by adding 1,10-phenanthroline to the imidazole-containing eluate prior to precipitation with ethanol. Single-stranded H_{6}-tagged templates prepared in this manner give uniformly high-quality DNA sequence data. Figure 5 shows the results of sequencing runs for all four single-stranded templates generated in this study, two of which (1b and 2b) represent unbound fractions from Ni^{2+}-NTA-agarose and two of which (1a and 2a) represent bound fractions eluted with 200 mM imidazole. DNA templates in Figure 4 are designated according to the PCR primer that they incorporate.

CONCLUSIONS

In this report, we have described a method for the affinity purification of DNA using immobilized metal affinity chromatography. The method centers on the attachment to DNA of a ligand tag consisting of six successive 6-histaminylpurine residues (H_{6}-tag), which mediates selective adsorption onto a Ni^{2+}-charged chelate resin. To incorporate the 6-histaminylpurine moiety into DNA, we relied on a post-synthetic method known as the convertible nucleoside approach (12–17); alternatively, the same operation could presumably be accomplished using standard DNA synthesis chemistry and a suitably protected dH phosphoramidite. The H moiety can be viewed as an adenine residue
Figure 4. Denaturing polyacrylamide gel electrophoresis analysis of DNA strand resolution using IMAC. Each panel of three lanes represents the results obtained using a pair of PCR primers, one of which (1a or 2a) contains an H$_6$-tag and the other of which (2b or 1b, respectively) is unmodified. C (lanes 1 and 4), controls showing the mixture of strands obtained directly by PCR, prior to IMAC resolution; U (lanes 2 and 5), unbound fractions from IMAC; B (lanes 3 and 6), bound fraction from IMAC, after elution with 200 mM imidazole.

Figure 5. Autoradiogram of $^{35}$S sequencing reactions using single-stranded template DNA generated by the described separation technique: 2b, unbound DNA from PCR/IMAC using primers 1a and 2b; 1a, bound DNA from PCR/IMAC using primers 1a and 2b; 1b, unbound DNA from PCR/IMAC using primers 1b and 2a; 2a, bound DNA from PCR/IMAC using primers 1b and 2a.

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