As a summary of this chapter, two tables are given. Table I lists the labeled nucleosides reported to be available by chemical syntheses. Table II lists the NMR studies using labeled DNA oligomers that were prepared by chemical syntheses.

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[12] Uniform $^{13}$C/$^{15}$N-Labeling of DNA by Tandem Repeat Amplification

By Milton H. Werner, Vineet Gupta, Lester J. Lambert, and Takashi Nagata

Introduction

Multidimensional heteronuclear nuclear magnetic resonance (NMR) has become a standard technique to determine the three-dimensional structure of proteins and RNA in solution. One of the most important advances in the application of NMR spectroscopy to the study of biological systems has been the ease of incorporation of $^{13}$C and/or $^{15}$N into proteins and RNA. The enrichment of macromolecules in these stable isotopes allows for the dispersion of $^1$H, $^{13}$C, and $^{15}$N chemical shifts into multiple spectral dimensions in a manner that preserves the chemical and/or spatial relationship between atoms within a molecule of interest.

Correlation of $^1$H resonances with their attached $^{13}$C and/or $^{15}$N chemical shifts enhances the spectral resolution and facilitates the analysis of macromolecular structure from both angular ($J$ coupling) and distance (nuclear Overhauser effect, NOE) restraints. Many of the experimental benefits of $^{13}$C and/or $^{15}$N enrichment realized for protein and RNA spectroscopy may also be applicable to analysis of DNA structure and its complexes with proteins. In pursuit of such a goal, three different approaches to the preparation of isotopically enriched DNA have been described in recent years.\textsuperscript{10–18} Solid-phase chemical synthesis using either phosphonate\textsuperscript{10} or phosphoramidite\textsuperscript{11,12} chemistry permits the greatest flexibility in labeling schemes, but may be technically demanding depending on circumstance. For this reason, enzymatic synthesis has become a more popular alternative for the preparation of uniformly labeled deoxyribonucleic acids.\textsuperscript{13–18}

Two fundamentally different enzymatic strategies have been described to date. The first approach, proposed by Zimmer and Crothers,\textsuperscript{13} employed a duplex DNA with a single-stranded overhang that served as a template for the synthesis of a complementary strand of user-specified sequence. Since its introduction, three different polymerases have been used to synthesize labeled DNA using this template-directed approach, each with its particular advantages: DNA polymerase Klenow,\textsuperscript{13,14} $Taq$ polymerase,\textsuperscript{15} and reverse transcriptase.\textsuperscript{18} An alternative enzymatic strategy proposed by Louis et al.\textsuperscript{16} employs a tandem repeat of user-defined sequence that can be amplified by thermal cycling. This second approach was originally developed to create long, tandemly repeated DNA sequences for the study of protein binding\textsuperscript{19,20} and for the production of hybridization probes,\textsuperscript{21} a technique known as the concatemer chain reaction.\textsuperscript{21} Irrespective of the method employed, enzymatic syntheses rely on the availability of milligram quantities of uniformly labeled $^{13}$C-and/or $^{15}$N-deoxynucleotide triphosphates (dNTPs), which must be prepared from the genomic DNA of a suitable microorganism.

\textsuperscript{11} A. Ono, S. Tate, and M. Kainosho, \textit{Tanpakushitsu Kakusan Koso} 40, 1509 (1995).
In this report, we describe detailed procedures for the large-scale synthesis of uniformly labeled duplex DNAs using polymerase chain reaction (PCR)-amplified, tandemly repeated templates of desired sequence. Because of the ready availability of labeled nucleic acids as a by-product of the production of labeled proteins expressed in *Escherichia coli*, our method focuses on the simultaneous preparation of labeled proteins and nucleic acids and their independent isolation from a single growth in this organism. We also describe the preparation of the kinasing enzymes and polymerase required for routine 10–50 mg scale synthesis of isotope-enriched DNA. The method is robust, providing a minimum amplification of 800-fold with essentially no synthetic by-products.

High Density Growth of *Escherichia coli* by Adaptive Control Fermentation

**Reagents**

*Escherichia coli* BL21 (47092, ATCC, Rockville, MD)

$[^{13}\text{C}]$ Glucose

$[^{15}\text{N}]$ Ammonium chloride

Minimal growth media

**Equipment**

Bench scale fermentor equipped with a mass flow analyzer, dissolved oxygen, pH, and temperature probes.

YSI 2000 glucose analyzer (optional)

Glucose test strips

**Procedure**

1. *Minimal growth media*. Prepare minimal growth media by autoclaving 6.8 g anhydrous sodium phosphate dibasic, 3.0 g anhydrous potassium phosphate monobasic, 0.5 g NaCl dissolved in 1 liter of deionized water. Separately prepare stock solutions by sterile filtration of the following: 1 $M$ MgSO$_4$, vitamin solution (5 mg/ml niacin, 5 mg/ml thiamin) (store at 4°C, good for 1 year), and 1000 $\times$ trace elements$^{22,23}$ (40.8 mM CaCl$_2 \cdot$H$_2$O, 21.6 mM FeSO$_4 \cdot$7H$_2$O, 5.8 mM MnCl$_2 \cdot$4H$_2$O, 3.4 mM CoCl$_2 \cdot$6H$_2$O, 2.4 mM ZnSO$_4 \cdot$7H$_2$O, 1.6 mM CuCl$_2 \cdot$H$_2$O, 0.64 mM boric acid, 0.2 mM (NH$_4$)$_6$Mo$_7$O$_{24} \cdot$4H$_2$O) (store in the dark at room temperature). Immediately prior to use, add to each liter of autoclaved


salts: 3 ml 1 M MgSO₄, 5 ml vitamin solution, 1 ml trace elements. For ¹⁵N labeling, add 2.0 g/liter of [¹⁵N]ammonium chloride and 12 ml of 50% (w/v) anhydrous glucose solution. For ¹³C/¹⁵N labeling, add 2.0 g/liter [¹⁵N]ammonium chloride and 5.0 g/liter [¹³C]glucose. Add the appropriate antibiotic if protein labeling is to be done simultaneously with nucleic acid labeling.

2. Take a small scratching of bacterial glycerol stock, inoculate a 1 ml culture in Luria broth (LB), and grow to saturation. Transfer 10 µl of this culture to 1 ml of minimal media and grow to saturation. The entire minimal saturated culture is then used to inoculate a 25 ml minimal culture and growth is continued for 6 more hours. The entire 25 ml minimal culture is then transferred to 1/5 volume of the desired final culture and grown overnight.

Note for ²H labeling: This protocol is also suitable for fractional or perdeuteration of proteins or nucleic acids. The level of deuteration using protonated glucose is approximately 18% less than the percentage of D₂O used to prepare the media. Therefore, for deuteration levels above 82%, [¹³C,²H₇]glucose must be used. For ²H labeling, the anhydrous phosphate salts are dissolved in D₂O, sterile filtered, and the remaining minimal media reagents added as dry solids directly to the D₂O–phosphate solution just before use. Trace elements may be added as an H₂O solution since they represent only 0.1% of the growth medium by volume. To adapt the cells to D₂O medium, a 1 ml minimal H₂O culture is first diluted 10-fold into a 1 ml minimal medium containing 50% D₂O (v/v) and grown to saturation. This 1 ml 50% D₂O culture is then diluted 50-fold into a 1 ml “100%” D₂O medium and grown to saturation. The remaining procedures for large-scale growth are the same as those described for minimal medium H₂O cultures.

3. Adaptive control fermentation. The objective of adaptive control fermentation is to maintain the bacterial culture in log phase growth for as long as possible to maximize the production of biomass from which nucleic acids or an overexpressed protein can be isolated (Fig. 1). To achieve this end, all of the nutrients are added to the medium at the beginning and the growth is monitored for pH, temperature, and dissolved oxygen continuously using a microprocessor-controlled measurement system (Fig. 1). The adaptive controller receives real-time reports on the dissolved oxygen level (pO₂) and attempts to hold the culture at 30% pO₂ by linear variation of both stir rate and air flow into the medium. To accomplish this task, a recursive least-squares method is employed based on the algorithm of Hsiao et al. To reach the target value for pO₂, the adaptive controller uses a regression calculation to “learn” what percentage change in stir rate and air flow is necessary to maintain the culture at the target level. Since pO₂ is evaluated every 15 ms, the controller responds effectively to the change in growth rate as the cell density increases. We have used this control mechanism to grow bacterial cultures to 20 A₆₀₀ units

FIG. 1. Schematic of an adaptive control bench-top fermentor. A water-jacketed vessel is linked to a 4°C water source with a computer-controlled heater. Individual measurement probes are inserted into the vessel to monitor temperature, stir rate, air flow, pH, and dissolved oxygen. The measured control parameters are digitized and fed into a Windows NT-based software package supplied by the fermentor manufacturer. The adaptive controller is a stand-alone module that receives the measured stir rate, air flow, and dissolved oxygen values from the NT computer and returns adjusted stir rate and air flow values necessary to maintain the target value for dissolved oxygen. This system has been implemented for both B. Braun Biotech DCU and New Brunswick BioFlow 2000 bench-top fermentation systems.

in minimal media and to >100 A_{600} units in LB in a single liter. The system is self-contained and requires minimal user input during the growth period. Because of the optimal growth condition under adaptive control, we routinely induce bacterial cultures at 3–5 A_{600} units for protein production, providing a 5- to 10-fold increase in biomass per unit volume. Off-line monitoring of carbon sources permits the quantitative consumption of labeling precursors to ensure maximal utilization (Fig. 2).

Preparation of Bulk Nucleic Acids

Reagents

Liquified phenol (Baker, Philipsburg, NJ)
Hydroxyquinoline (Fluka, Ronkonkoma, NY)
FIG. 2. Time course of growth parameters during adaptive control fermentation. (a) The various control parameters are monitored in real time and plotted to follow the time course of growth of the culture. The learning phase (0–0.5 hr) is accompanied by a large oscillation in the target function (pO2) as the controller "learns" how to vary stir rate and air flow to hold the culture at 30% pO2. Once the variation needed is learned, a linear increase in these parameters is observed. The oscillations in pO2 dampen as the culture density increases. At the point of protein expression induction, the culture slows cellular replication, resulting in a plateau in the measured growth parameters, consistent with the switch from a growth to an expression phase for an inducible protein. When applied to the labeling of nucleic acids alone, the culture will continue in log-phase growth to very high densities (>20 A600) as long as nutrients (carbon and nitrogen sources) remain in the medium. (b) Variation in optical density and glucose consumption during adaptive control fermentation. The consumption of glucose in this example was approximately 5 g/liter in a 1.2 liter culture grown to 8 A600 units.
Chloroform
Isoamyl alcohol
100% Ethanol (USP grade)
3 M Sodium acetate, pH 5.3
Lysis buffer:

Nucleic acids only: 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM Na₂EDTA
Nucleic acids + soluble His-tagged protein: 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 10 mM benzamidine hydrochloride
Nucleic acids + soluble untagged protein: 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM benzamidine hydrochloride, 1 mM dithiothreitol (DTT)
Lysozyme (Sigma, St. Louis, MO)
Pronase (Sigma)
Hydrated diethyl ether: Diethyl ether treated with an equal volume of 50 mM Tris-HCl, 1 mM Na₂EDTA. Aqueous layer (bottom) is removed before use.

Equipment

Homogenizer (Fisher Power Gen 700)
500 ml separatory funnel
French press
1000 Molecular weight cutoff (MWCO) dialysis membrane

Procedure

1. Prepare phenol. Equilibrate phenol according to established procedures.²⁵ Mix equilibrated phenol, chloroform, and isoamyl alcohol in a 25 : 24 : 1 (v : v : v) ratio. This may be stored at 4 ° in an amber bottle for 1–2 months.

2. Homogenize cell paste. Volumes indicated are suitable for the preparation of nucleic acids from 35 g of wet cells and may be scaled linearly for different amounts of cell paste. The cells are suspended with a homogenizer in 4 ml lysis buffer/gram wet cell paste until a smooth slurry is created. The choice of lysis buffer is dependent on whether proteins are to be simultaneously isolated and by what method; the buffer composition for several common examples is described under Reagents.

3. Process lysate. (a) For nucleic acids only: The homogenized cells are passed through a French press twice at 500–1000 psi, 1 mg/ml lysozyme is added, and the lysate is incubated with nutation at 37 ° for 1 hr. Pronase (1.5 mg/ml) is added and the lysate incubated with nutation at 37 ° a further 8–12 hrs.²⁶,²⁷ Sodium dodecyl

²⁷ M. G. Smith, Methods Enzymol. 12, 545 (1967).
sulfate (SDS) (10% w/v) is added with gentle stirring until the opaque, caramel-colored lysate becomes translucent. One volume phenol : chloroform : isoamyl alcohol is added and stirred at room temperature for 30 min in a 500 ml centrifuge bottle in a chemical hood. (Phenol is caustic and appropriate safety precautions should be observed.) Spin the phenolic solution at 5000 g for 10 min and transfer the top (aqueous) layer with a glass pipette into a new centrifuge bottle. This aqueous layer will be a translucent, pinkish solution. Reextract the phenolic layer with a second equal volume of lysis buffer, shake vigorously, and centrifuge. Combine two aqueous layers and add an equal volume of hydrated diethyl ether, shake briefly, and centrifuge. Recover aqueous layer (bottom) with a separatory funnel. Add 1/10 volume 3 M sodium acetate (pH 5.3) and 3 volumes 100% ethanol to precipitate nucleic acids. Allow precipitation to continue for at least 12 hr at -20°C.

(b) For nucleic acids + soluble His-tagged proteins: Cells are homogenized and French pressed in the appropriate lysis buffer (see Reagents) and centrifuged at 5000 g. The pellet is kept frozen until recombined with the soluble nucleic acid fraction as described below. The supernatant is centrifuged at 100,000 g in a preparative ultracentrifuge at 4°C for 1 hr and subsequently passed over Ni²⁺-chelate affinity resin after adding imidazole to the supernatant to 30 mM. The flow-through of the affinity column, which contains the nucleic acids, is combined with the membrane pellet, lyophilized, and resuspended with homogenization in the lysis buffer described in section (a), for nucleic acids only, and processed as described above.

(c) For nucleic acids + soluble untagged proteins: Cells are homogenized and French pressed in the appropriate lysis buffer (see Reagents) and centrifuged at 5000 g. The pellet is kept frozen until recombined with the soluble nucleic acid fraction as described below. NaCl is added to the supernatant to a final concentration of 500 mM and passed over anion-exchange resin (Q-sepharose) at 2 ml/min (2.6 x 40 cm). Elution of the nucleic acid fraction is achieved with a linear gradient of 5 column volumes from 500 mM to 1.2 M NaCl in 50 mM Tris-HCl (pH 8.0). The eluted nucleic acids are then lyophilized, combined with the membrane pellet, and treated identically as described above. The desired protein from the flow-through of the anion-exchange column can be subsequently purified with a user-defined protocol following removal of excess salts by dialysis.

Notes on preparation of lysate. Most of the chromosomal DNA is membrane-bound following French press. The use of lysozyme and pronase solubilizes the DNA, permitting quantitative recovery of this nucleic acid fraction. Pronase, a mixture of three aggressive endoproteinases from Streptomyces griseus, will completely digest all proteins present in the lysate. Failure to use lysozyme and/or pronase will hinder the ability to recover the DNA reproducibly. Heating the phenolic solution or blending the phenolic solution is less reliable for DNA recovery in our experience. Enzyme treatment also has an added advantage when large scale (>20 g) extraction of cells is performed. Following phenolic extraction, a pronase-treated lysate will display only a thin white interface layer.
that is no more than a few millimeters thick. This improves the ease with which the aqueous layer can be recovered with a glass pipette. Stirring in SDS prior to phenolic extraction also helps in the recovery of the nucleic acid layer by shearing the chromosomal DNA, thereby reducing the viscosity of the aqueous layer following separation of the phenolic solution by centrifugation. It is imperative to add sufficient SDS that the lysate is completely clarified prior to phenolic extraction; otherwise, lipid and protein impurities will be carried along with the DNA that are not as easily removed in subsequent steps.

4. Prepare nucleic acids for digestion. The precipitated nucleic acids are centrifuged at 10,000g for 1 hr and the supernatant carefully decanted. The slightly yellowish pellet is air dried, resuspended in 75–125 ml 10 mM Tris-HCl, pH 8.0, and dialyzed in 1000 MWCO tubing against 4 liters of the same buffer for 8–12 hr at room temperature. At least two changes of this buffer should be done during dialysis to remove nonnucleic acid impurities and residual diethyl ether. The nucleic acids are subsequently centrifuged at 100,000g for 1 hr and the pH of the supernatant adjusted with NaOH to 7.5–8.0. Using the procedures outlined above, we routinely recover 28–30 mg nucleic acid/gram of wet cells following dialysis. (Because the nucleic acids are largely RNA, we assume a concentration of 40 µg/ml per A\textsubscript{260} unit for the resuspended nucleic acids following dialysis.)

Preparation of Deoxynucleotide Monophosphates

Reagents

RNase-free DNase I (Boehringer-Mannheim)
Nuclease P1 (Sigma)
RNase A (Pharmacia, Piscataway, NJ)
MgSO\textsubscript{4}
ZnSO\textsubscript{4}
Affi-Gel 601 (Bio-Rad, Hercules, CA)
1 M Triethylammonium bicarbonate (TEABC)
1 M Triethylammonium acetate (TEAA)
1 M Ammonium bicarbonate (ABC, optional)

Equipment

5.0 × 20 cm medium pressure chromatography column
25 × 250 mm C\textsubscript{18} reversed-phase (RP) semipreparative HPLC column
(Varian)

Procedures

1. Digestion of duplex DNA. The bulk nucleic acids are digested for 2 hr at 37\degree, pH 8.0, by the addition of 1 unit RNase-free DNase I per 660 µg total nucleic acid.
DNase I is shock sensitive, so very slow nutation of the nucleic acid solution should be used. DNA digestion is monitored by 0.7% (w/v) agarose gel electrophoresis. There should be no nucleic acid species binding ethidium bromide greater than 5000 base pairs prior to the next step, otherwise duplex DNA digestion could be incomplete. If necessary, add an additional aliquot of DNase I and incubate a second time.

2. Digestion of RNA and single-strand DNA. Following duplex DNA digestion, the nucleic acids are denatured by heating in a water bath at 95° for 10 min and rapidly chilled on ice. One unit of nuclease P1 is added per 24 mg total nucleic acid. Digestion is carried out at pH 8.0, 55°, for 2 hr, and the nucleic acids are again denatured and rapidly cooled on ice. A second, equal amount of Nuclease P1 is added without denaturation and digestion allowed to proceed for 2 more hr at 55°. At this point, 0.7% agarose gel electrophoresis should reveal the presence of only a minor band of ethidium bromide stainable nucleic acid of 100–200 base pairs in length. This material is resistant to mung bean nuclease, nuclease S1, exonucleases I and III, and Bal31 nuclease. It is easily digested with the addition of RNase A, suggesting that it is RNA (most likely tRNA given its size). One mg RNase A per 80 mg total nucleic acid is sufficient to completely digest this material within 2 hr at 37°.

Note on RNA digestion: We routinely observe a nuclease P1-resistant RNA species in E. coli nucleic acid preparations irrespective of the pH used, quantity of enzyme, or length of time allowed for digestion. This resistant RNA is present whether or not the cells are grown to saturation. If the nucleic acids are not denatured following the second P1 digestion, P1 will remove the 3'-phosphate from the mononucleotides released by RNase A. Although the product of RNase A/nuclease P1 digestion are ribonucleosides, they form only a minute fraction of the total RNA-derived products. It is essential that there be no ethidium bromide stainable nucleic acids remaining prior to separation of dNMPs from rNMPs by boronate affinity chromatography. Undigested nucleic acids, whether DNA or RNA, do not stick to the boronate affinity resin under the conditions described below, contaminating the dNMP fraction.

3. Separation of dNMPs from rNMPs. The dNMPs are separated from rNMPs by boronate affinity chromatography (Affi-Gel 601) as previously described. To prepare the column, swell the resin in 20 volumes of cold, CO2-acidified water (pH 4–4.5) and pack a 5.0 x 20 cm column at 1.0 ml/min, 4°. Once the bed height has stabilized, equilibrate the column with 150 ml 1 M TEABC at 4°. The column resin will shrink to one-half its original height. Do not readjust the column adapter since elution is conducted at acid pH which will reswell the gel. Fifteen g Affi-Gel 601 will efficiently separate the nucleotide monophosphates derived from 500 mg of bulk nucleic acid.

DIGESTION AND PURIFICATION OF dNMPs.

(a) RP-HPLC chromatogram of dNMPs following boronate affinity chromatography. The dNMPs are relatively pure, with a few minor products that represent small single-stranded nucleotide fragments resistant to digestion. (b) Preparative RP-HPLC of dNMP fraction following boronate affinity chromatography. The flow-through fractions from boronate affinity chromatography are lyophilized and resuspended in 20 ml 0.1 M TEAA. Five ml is then injected and purified by RP-HPLC as described in the text. The individual nucleotides are collected and lyophilized prior to phosphorylation. Reinsertion of preparative RP-HPLC dNMPs on an analytical 4.6 × 25 cm RP-HPLC column reveals each nucleotide solution to be >98% pure.

The digested nucleic acids are dried by centrifugal lyophilization and resuspended in cold 1 M TEABC. Chill the nucleotide solution and pump onto the column at 4°C, 0.5 ml/min. The column is washed with 1 M TEABC, 4°C, 0.5 ml/min, until the A254 returns to baseline on the detector (250 ml for a column prepared from 15 g Affi-Gel 601). The flow-through fractions, containing the dNMPs, are combined and lyophilized. The rNMPs are eluted by washing the column with 250 ml CO2-acidified water (pH 4-4.5). Purity of the nucleotide monophosphates can be assessed by C18 reversed-phase HPLC (RP-HPLC) in 100 mM TEAA pH 6.8 using a linear gradient of 2-18% methanol, 10 ml/min for 60 min (Fig. 3). Preparative purification of individual nucleotides can be accomplished with the same gradient (Fig. 3). Typical yields are 90-100 mg each dCMP, dAMP, dGMP, and 60-80 mg of dTMP after preparative RP-HPLC of processed nucleic acids derived from 35 g wet cell paste.

Notes: TEABC can be replaced by 1 M ammonium bicarbonate (ABC), pH 9.5. The advantage of ABC is its easier removal by lyophilization relative to TEABC. (One M TEABC is prepared by stirring 141 ml HPLC-grade triethylamine in 859 ml deionized water on ice in a chemical hood. CO2 is bubbled into the mixture with vigorous stirring until most of the triethylamine is dissolved in the water. The pH is then monitored with further bubbling until it reaches 9-9.5. One M TEAA stock solution can be prepared by mixing 141 ml HPLC-grade triethylamine, 57 ml
glacial acetic acid, and 750 ml deionized water at room temperature in a chemical hood. The pH is then adjusted with additional glacial acetic acid until it reaches pH 6.8. The stock solution is filtered and stable at room temperature for at least 1 year.)

Preparation of Deoxynucleotide Triphosphates

Reagents

- Yeast thymidylate kinase (TMPK) (laboratory prepared)
- E. coli Cytidylate monophosphate kinase (CMPK) (laboratory prepared)
- Myokinase (MK) (Sigma)
- Pyruvate kinase (PK) (Sigma)
- Guanylate kinase (GK) (Sigma)
- Phospho(enol)pyruvate (PEP) (Sigma)
- 10x Phosphorylation buffer (800 mM Tris-HCl, pH 7.5; 200 mM KCl, 200 mM MgCl₂)
- Benzamidine hydrochloride
- Glutathione agarose (Sigma)
- Blue Sepharose (Pharmacia)

Equipment

- 1.6 x 20 cm medium pressure column
- 2.6 x 20 cm medium pressure column

Procedure

1. Preparation of TMPK. All procedures should be carried out at 4°C. Yeast TMPK is expressed as a glutathione transferase (GST) fusion and grown by conventional methods. Cells are disrupted by French press in 20 mM sodium phosphate, pH 7, 150 mM NaCl, 10 mM benzamidine hydrochloride. Triton X-100 is added to 1% (v/v) and the lysate clarified by centrifugation at 5000g. The supernatant is ultracentrifuged at 100,000g for 1 hr and loaded onto a 1.6 x 15 cm glutathione–agarose column at 1 ml/min. The column is extensively washed with 20 mM sodium phosphate, pH 7, 150 mM sodium chloride until the absorbance at 280 nm returns to baseline. The protein is then eluted isocratically with 50 mM Tris-HCl, pH 8, 100 mM NaCl, 15 mM reduced glutathione, 1 mM DTT, 0.05% (v/v) nonidet P-40 (NP-40). The eluted protein is extensively dialyzed against 25 mM HEPES–KOH, pH 7.4, 100 mM NaCl, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% (v/v) glycerol, 0.05% NP-40. The protein is concentrated to 1.7 mg/ml and stored in aliquots at -80°C.
2. Preparation of CMPK. All procedures should be carried out at 4 °C. E. coli CMPK is expressed by conventional methods in pET22b as described. Cells are disrupted by French press in 50 mM Tris-HCl, pH 7.5, 10 mM benzamidine hydrochloride, and clarified by centrifugation at 5000g. The supernatant is subsequently centrifuged at 100,000g and loaded onto a 2.6 x 15 cm Blue Sepharose column equilibrated with 50 mM Tris-HCl, pH 7.5. The column is washed until the absorbance at 280 nm is returned to baseline, and the protein is eluted isocratically with 50 mM Tris-HCl, pH 7.5, 1.2 M NaCl. The eluted protein is extensively dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM DTT. The protein is concentrated by ultrafiltration to 2.5 mg/ml, glycerol is added to 50% (v/v), and the samples are stored in aliquots at -20 °C.

3. Enzymatic phosphorylation. The individually purified dNMPs are converted >95% to their corresponding dNTPs in four individual reactions by a modification of established procedures, to include the utilization of recombinant TMPK and CMPK to phosphorylate dTMP and dCMP, respectively. Myokinase (MK, final concentration = 1 unit/µl) and guanylate kinase (GK, final concentration = 0.1 unit/µl) are dissolved in degassed 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 50% glycerol (v/v), and stored in a screw-cap vial at -20 °C. TMPK and CMPK are prepared under similar conditions as described above. The phosphorylation reaction for dAMP, dCMP, and dGMP contains 1× phosphorylation buffer, 10 mM PEP, 1 mM dNMP. TMPK efficiently catalyzes the conversion of dTMP to dTTP; therefore, PEP and PK are excluded from this reaction. Kinasing enzymes and rATP are added to a final concentration as follows: 69 units MK/mg dAMP, 28 units PK/mg dAMP, 0.1 mM rATP; 90 µg CMPK/mg dCMP, 28 units PK/mg dCMP, 0.1 mM rATP; 0.46 units GK/mg dGMP, 28 units PK/mg dGMP, 0.5 mM rATP; 90 µg TMPK/mg dTMP, 5 mM rATP. The reaction is assembled in the following order: buffer, dNMP, PEP, rATP, PK, and finally the appropriate nucleotide monophosphate kinase. Prior to the addition of enzymes, the reaction solution is extensively sparged with argon. The reaction is incubated at 37 °C for 8 hr (dAMP, dCMP), 12 hr (dTMP), or 20 hr (dGMP) with the conversion monitored by analytical RP-HPLC using a 0→20% gradient of 98 mM TEAA, 2% CH3OH vs 78 mM TEAA, 30% CH3OH, pH 6.7, over 30 min at 0.5 ml/min. For 40 × 500 µl reactions, 3.3–3.9 mg of each dNTP is required (Fig. 4).

Following phosphorylation, the reaction is quenched by the addition of Na2EDTA to 20 mM and the dNTPs purified by semipreparative C18 RP-HPLC

Fig. 4. Conversion of dNMPs to dNTPs. Analytical RP-HPLC chromatograms of the beginning and end-point composition of enzymatic phosphorylation reactions. For each pair of chromatograms, the zero time point appears in the foreground and the end point appears in the background, offset diagonally to demonstrate the nearly complete conversion of dNMP to dNTP. Note that for dTMP→dTTP, a 25-fold greater concentration of rATP is used. At this higher concentration, rAMP and rADP are observed in the dTMP zero time-point chromatogram because of spontaneous dephosphorylation of the rATP stock solution. For dCMP→dCTP, the PEP retention time is coincident with that of dCMP itself, accounting for the presence of a small peak in the dCTP chromatogram.

using the conditions described under “Preparation of deoxynucleotide monophosphates.” The purified dNTPs are concentrated by lyophilization and stored at −80°.

PCR Amplification of Tandemly Repeated Oligonucleotides

Reagents

Pfu DNA polymerase (ATCC 87496)
Whatman P11 phosphocellulose
Anion-exchange resin
Polyethyleneimine (Fluka)
(NH₄)₂SO₄ (ultrapure enzyme grade)
10 × Pfu PCR buffer: 200 mM Tris-HCl, pH 8.8, 100 mM KCl, 100 mM (NH₄)SO₄, 1.0% Triton X-100, 1.2 mg/ml bovine serum albumin (BSA)
100 mM MgSO₄
20 mM labeled dNTPs
Gel-purified oligonucleotide templates
EcoRV (Promega, Madison, WI)

**Equipment**

- Thermal cycler (non-Peltier model preferred)
- 2.6 × 20 cm medium pressure chromatography column
- 2.5 × 10 cm Vydac 301 VHP DEAE HPLC column

**Procedures**

1. **Preparation of Pfu polymerase.** The expression vector for Pfu polymerase is transformed into *E. coli* HMS174(DE3) pLysS using standard procedures.²⁵ The cells can be grown by conventional methods and induced with 0.4 mM IPTG for 4 hr. The cells are harvested by centrifugation at 5000g and lysed in 50 mM Tris-HCl, 1 M NaCl, 10 mM Na₂EDTA, 10 mM benzamidine hydrochloride using the homogenization/French press approach described under "Preparation of bulk nucleic acids." Unless indicated otherwise, all subsequent steps should be performed at 4°C. The lysate is clarified by centrifugation at 5000g and the nucleic acids precipitated by the addition of 7.5 ml 10% (v/v) polyethyleneimine per 100 ml lysate. The white precipitate is removed by centrifugation at 5000g. The supernatant is transferred to a 500 ml centrifuge bottle and heated in a water bath at 74°C for 20 min, then rapidly chilled on ice. Most *E. coli* proteins will be precipitated at this stage and can be removed by centrifugation. Pfu polymerase is then precipitated by adding solid ammonium sulfate to 85% saturation. The protein precipitate is recovered by centrifugation at 10,000g, resuspended in 50 mM Tris-HCl, pH 7.5, 1 mM Na₂EDTA, 10 mM benzamidine hydrochloride and dialyzed against 4 liters of the same at 4°C for at least 12 hr. The protein solution is centrifuged at 100,000g for 1 hr and loaded at 1.5 ml/min onto a 2.6 × 20 cm column of Whatman P11 (phosphocellulose) equilibrated with 50 mM Tris-HCl, pH 7.5. Pfu polymerase is eluted with a two column volume gradient from 0→1.2 M NaCl. Pfu polymerase elutes at approximately 180 mM NaCl. The eluted fractions are pooled, dialyzed against 10 mM Tris-HCl, pH 8.0, until the measured conductivity of the protein solution is within twofold of the buffer. Pfu is further purified by Mono Q (1.6 × 20 cm) (or any quaternary anion-exchange resin) using a 5 column volume linear gradient from 0 to 18% 1 M KCl. Pfu polymerase elutes in three chromatographically distinct peaks, with the most abundant fraction retained for
use as a thermal cycling enzyme. The main fraction is pooled and dialyzed against 10 mM Tris-HCl, pH 8.2, 0.2 mM EDTA extensively. The protein solution is then analyzed for purity by SDS-PAGE and concentrated by ultrafiltration to 1.3 mg/ml. Nuclease digestion assays should be performed to confirm that no nuclease has copurified with the enzyme.25 The protein is then diluted 2× with 2 mM DTT, 0.2% (v/v) NP-40, 0.2% (v/v) Tween 80, 100% (w/v) glycerol, and aliquoted for storage at −20°C. Long-term storage can be at −80°C, but the enzyme should not be repeatedly thawed and refrozen at this temperature. One μl of a fivefold dilution of enzyme is typically adequate for standard (cloning-type) PCR reactions. Undiluted enzyme is used for the thermal cycling procedure described below.

2. PCR amplification of tandemly repeated oligonucleotide templates. DNA synthesis is conducted in 40 × 500 μl thin-walled reaction vials in a gradient thermal cycler equipped with a “hot” top to avoid the use of mineral oil. A tandem repeat of the desired sequence is synthesized at the 1 μmol scale without trityl groups and gel purified on sequencing length gels.25 The two step reaction procedure is similar in design to the concatemer chain reaction of Thompson and co-workers21 as well as the adaptation of this approach to DNA-labeling by Louis et al.,16 with the exception that the anealing temperature in each step is optimized for each template sequence using an optimized cycling protocol (Figs. 5 and 6). In step 1, the anealing temperature is optimized by systematic variation over 14°C and examining the total nucleic acid content and length distribution of the product DNA by 0.7% agarose gel electrophoresis (Fig. 6a and Table I). For step 2, the annealing temperature is optimized over a similar temperature range by quantifying the digested DNA product in 15% polyacrylamide gels (0.5× TBE) (Fig. 6b and Table I).

A typical step 1 reaction contains 0.1 μM each of gel purified template, 2 mM labeled dNTPs (0.5 mM each nucleotide), 4 mM MgSO4, 1× Pfu reaction buffer, and 3.25 μg recombinant Pfu DNA polymerase. Thermal cycling procedure: 95°C for 5 min, 25 cycles of 95°C for 45 s, T1 for 2 min (see Table I), and 72°C for 4 min.

A typical step 2 reaction contains 50 μl step 1 product, 2 mM dNTPs, 4 mM MgSO4, 1× Pfu reaction buffer, 3.25 μg recombinant Pfu DNA polymerase. Single repeat DNA (~35 pmol) derived from restriction of step 1 reaction products or by restriction of the original templates may be added in step 2 to enhance overall product yield by as much as twofold (Figs. 5 and 6). Thermal cycling procedure: 95°C for 5 min followed by 60 cycles of 95°C for 45 s, T2 for 2 min (see Table I), and 78°C for 4 min. Thermal cycling was followed by incubation at 78°C for 10 min.

Note on choice of thermal cycler: Although the PCR cycling scheme outlined above will work in any thermal cycler, Peltier-driven machines were found to be relatively inefficient at amplification of tandemly repeated templates. We recommend the use of a cycler that does not ramp temperature in a single block, such as the Stratagene (La Jolla, CA) Robocycler 40. For optimization of a Peltier-driven machine using 500 μl tubes, it will be necessary to actually optimize the ramping times and rates in addition to the temperature of each step in the cycle. The conditions
FIG. 5. PCR amplification of tandemly repeated templates. A two-step synthesis is employed,\textsuperscript{16,21} the first of which prepares a self-priming/self-propagating template pool and the second of which synthesizes long, tandemly repeated DNA. The course of synthesis is followed by 0.7% agarose gel electrophoresis (right). (a) A tandem repeat of the desired sequence is added to the reaction mixture as a blunt-ended duplex. (b) Thermal cycling converts the blunt-ended duplex into a self-priming repeat, creating a pool of different length DNAs. (c) Step 1 products serve as templates for a second round of amplification. The step 1 products are diluted 10-fold into a series of step 2 reactions that create long tandem repeats. At the beginning of step 2, additional duplex DNA containing a single repeat of the desired sequence can be added to increase the overall yield by as much as twofold. Extensive thermal cycling (d) followed by restriction with \textit{EcoRV} (e) results in milligram quantities of single-length DNA product of the desired sequence.

described are the results of extensive optimization on a non-temperature-ramping thermal cycler. Under the described conditions, product yields are dependent on neither sequence composition nor length.

3. Endonuclease cleavage. The product DNA from 40 reactions is combined, DTT added to 1 mM, NaCl added to 125 mM, and MgCl\textsubscript{2} added to a total final concentration of 10 mM. The pH is lowered to 7.9 with HCl and the DNA digested with 150 units of \textit{EcoRV} per 500 \textmu l step 2 reaction at 37\textdegree C with continual mixing for 4 hr. Another 150 units of \textit{EcoRV} is added for an additional incubation of no more than 4 hr. Digestion is monitored by 15\% polyacrylamide gels (0.5× TBE) (Figs. 6 and 8).

\textit{Note:} The choice of \textit{EcoRV} here is essentially one of cost. Any restriction endonuclease may be used here depending on the properties of the ends that are desired.

4. Purification of product DNA and recovery of unincorporated nucleotides. Digested DNA is 0.2 \mu m filtered and purified by DEAE ion-exchange HPLC using a preparative Vydac 301 VHP column (Fig. 7). The column is equilibrated with 25 mM sodium phosphate, pH 7.4, 90 mM NaCl. The digested product DNA
FIG. 6. Optimization of synthetic yield by gradient thermal cycling. An example of the annealing temperature optimization procedure for template 2 involves two different tests that can be performed with unlabeled dNTPs. (a) Optimization of step 1 annealing temperature. Ten μl of 500 μl step 1 reactions are analyzed with 0.7% agarose gels that display the length distribution and total quantity of nucleic acid at different annealing temperatures. The bar graph quantitates the total fluorescent intensity, expressed in fluorescent “counts” in a Molecular Dynamics Storm System, illustrating that the total nucleic acid content is roughly equal at T1 = 49°C, 51°C, and 57°C. However, the length distribution varies widely at these three annealing temperatures as evidenced by the extent of smearing in the gel lanes. It is preferable to use the annealing temperature that displays a wide length distribution and maximal total nucleic acid content so that efficient priming in step 2 occurs. For this reason, the T1 = 49°C nucleic acid pool was chosen for further amplification in step 2. (b) Optimization of step 2 annealing temperature. Ten μl of 500 μl reaction is analyzed with 15% polyacrylamide, 0.5x TBE gels following EcoRV digestion as described in the text. For T1 = 49°C, priming in step 2 is more efficient overall when compared to T1 = 55°C as evidenced by the higher product yield in three of the four reactions shown. This reflects not only the higher total nucleic acid content of the T1 = 49°C reaction relative to T1 = 55°C, but also the greater dispersion in product lengths at T1 = 49°C. The greatest product yield is observed for the T1 = 49°C/2°C = 65°C combination in this example (see Table 1).

is diluted with phosphate buffer to lower the initial [NaCl] to ≤90 mM, injected (5 ml aliquots), washed over the column to remove unincorporated nucleotides, then eluted using a gradient of 90 mM→360 mM NaCl over 15 min at 10 ml/min. Fractions containing the main DNA peak were collected and dialyzed against 1 mM sodium phosphate, pH 7.0, 1 mM Na2EDTA and concentrated by lyophilization. The yield of product DNA was determined by measuring the absorbance at 260 nm assuming 50 μg/ml per A260 unit. A 500 μL reaction yields a minimum of 8.5 nmol of isotopically enriched single-length DNA from 5 pmol of unlabeled template, an 800:1 product:template yield. The pool of unincorporated nucleotides can be desalted by preparative C18 RP-HPLC column as described above, lyophilized, and stored at −80°C for rephosphorylation and reuse.
# TABLE I
DNA Templates and Target Sequences for ULTRA

<table>
<thead>
<tr>
<th>DNA template</th>
<th>Product sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCAGGATGCGTTACTGATATCAGGATGCGTTACTGAT</td>
<td>ATCAGGATGCGTTACTGAT</td>
</tr>
<tr>
<td>TAGTCTACTGCAATGACCTAGTCTACTGCAATGACCTAGCTAGTCCTACGCCAATGACTATAGTCCTACGCCAATGACTA</td>
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<td>ATCGTTTGTCGAT</td>
</tr>
<tr>
<td>TAGCAAAACAGCTATAGCAAAACAGCTA</td>
<td>TAGCAAAACAGCTATAGCAAAACAGCTA</td>
</tr>
</tbody>
</table>

ULTRA: Uniform Labeling by Tandem Repeat Amplification. EcoRV restriction site is shown in boldface type, a half-site at each end and full site separating the tandem repeat of desired sequence.

5. *Fill-in of overdigested product.* Occasionally, incubation of product DNA with EcoRV longer than 12 hr results in overdigestion, creating multiple product lengths. Overdigestion results in recessed 3' ends that can be filled-in with Klenow DNA polymerase and labeled dNTPs (Fig. 8). A typical fill-in reaction contains 0.1 mM DNA, 10 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 7.5 mM DTT, 2 mM dNTP mixture, and 50 units/ml Klenow. Fill-in is complete within 2 hr at 37°C. The reaction

![Figure 7](image-url)

**FIG. 7. Single-step purification of product DNA.** The restricted product DNA can be purified in a single step by DEAE ion-exchange HPLC in 25 mM sodium phosphate employing a biphasic gradient. Isocratic elution of a 5 ml injection at 90 mM NaCl separates the unreacted nucleotides from the product, permitting their recovery and reuse. A linear gradient over 15 min from 90 to 360 mM NaCl elutes the product DNA as essentially a single peak.
FIG. 8. Purity of product DNA and fill-in of overdigested product. (a) Purity of product DNA. 20% urea–polyacrylamide gel of product DNAs demonstrates that the product is identical in length to that derived from digested template DNA. There are two bands observed for this product, each of which represents one of the strands of the duplex DNA that are resolved in a 20% sequencing-length gel. We frequently observe a faint slower mobility band in these gels that represents product digested to only dimer length (<0.1%). The faint ladder seen below the product bands results from slight overdigestion and represents <0.3% of the sample. (b) Fill-in of intentionally overdigested product. Product DNA was digested for 14 hr, revealing substantial (~20%) overdigestion to a length 1–2 nucleotides shorter than the main product. Fill-in with labeled dNTPs and Klenow results in only full-length product plus the small fraction of dimer as described in (a). The dimer can be removed by gel filtration, if desired.
is quenched by the addition of Na$_2$EDTA to 10 mM and the DNA recovered by ion-exchange chromatography as described above.

Sequence Fidelity of Product DNA

The sequence fidelity and extent of isotopic enrichment can be assessed by mass spectrometry. However, we find it to be easier to assess the spectroscopic homogeneity of the product by two simple experiments. The composition of the product DNA can be determined from a constant-time $^{13}$C--$^1$H HSQC experiment (Fig. 9).$^{32}$ Simply counting the number of H$_5$–C$_5$ cross peaks of cytidine residues and the H$_2$–C$_2$ cross peaks of adenine residues will permit the direct assessment of the correct number of CG and AT base pairs, respectively. The digested product from template 2 (see Table I), for example, displays 11 adenine H$_2$–C$_2$ correlations (two cross peaks have two-proton intensities) and 9 cytidine H$_5$–C$_5$ correlations (one cross peak has two-proton intensity) as expected. The symmetry-related nucleotides in the EcoRV half-sites at each end of the product duplex are magnetically equivalent (Fig. 9). An additional verification of sequence composition can be accomplished by counting the number of cross peaks of a specific residue type in a constant-time multiple-quantum Hb(Cb)Nb experiment which correlates the base proton (Hb) to the N9 or N1 nitrogen (Nb) via the C8 or C6 base carbon (Cb) (Fig. 9b).$^{33}$ These spectroscopic analyses coupled with assessment of the purity of the digested DNA product by denaturing urea–PAGE are sufficient to be confident that the product is of high purity and homogeneity.

Summary

An optimized procedure has been described for the large-scale production of stable isotopeenriched duplex oligonucleotides of designed sequence. Large-scale production of labeled nucleotide triphosphates can be produced in this procedure simultaneously with labeled proteins, thereby providing synthetic dNMP precursors at no additional cost. The procedure is robust, with a minimum product : template yield of 800 : 1 overall, and produces >99% single-length product. Tandem repeat PCR amplification is a general approach to large scale synthesis of duplex oligonucleotides and may have applications to both NMR and X-ray methods, particularly for product lengths in excess of 25 base pairs where failed sequences from solid-phase synthesis can be difficult to remove chromatographically. A drawback of the present approach is that the product is a duplex of two equal-length strands, making single-stranded products more difficult to prepare. For this reason, it could be preferable to produce single-stranded products by the


FIG. 9. Sequence fidelity of DNA products. (a) The sequence fidelity can be assessed in a single constant-time \(^{13}\text{C}-^{1}\text{H} \) HSQC spectrum. \(^{32}\) Sequence 2 contains 9 GC and 11 AT basepairs (see Table I), which can be verified by counting the number of cytidine C5–H5 and adenine C2–H2 cross peaks. Symmetry-related nucleotides in the EcoRV half-sites at each end of the duplex are magnetically equivalent and are indicated with asterisks in the figure. (b) Residue-type assignment of the nucleotides of sequence 2 by a constant-time Hb(Cb)Nb experiment. \(^{33}\) The nucleotides group by residue type according to their N9 (purine) or N1 (pyrimidine) chemical shifts. Magnetically equivalent residues are indicated by asterisks. For T20/T40, there is an alternate conformer seen at H6 = 7.65 ppm/\(^{15}\text{N} = 146.5 \) ppm. This is most likely due to fraying at the ends of the sequence. The remainder of the spectrum is nearly fully resolved for every nucleotide. The spectra were collected at 600 MHz, 36°, in pH 7 phosphate buffer as described. \(^{32,33}\)

method of Zimmer and Crothers. \(^{13-15}\) Although a single base type can be selectively enriched in this approach, chemical synthesis will provide greater flexibility for labeled DNAs requiring site-specific labels at only one or a small number of nucleotide positions in the sequence. \(^{10-12}\) Therefore, maximum flexibility in labeling patterns can be realized by judicious choice of labeling method appropriate to the type of DNA product and extent of isotopic enrichment desired.

1-ATCAGGATGCCTACTGAT-20
40-TAGTCCTACGCCAATGACTA-21