Automated Two-Column Purification of Iminobiotin and BrdU-Labeled PCR Products for Rapid Cloning: Application to Genes Synthesized by Polymerase Chain Assembly

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Abstract

Polymerase chain assembly (PCA) is a powerful tool for basic biological research and biotechnology applications. During the last several years, major advances have been made in *de novo* gene synthesis. However, there is still a need for fast and reproducible methods to automatically purify the synthesized genes. Upon completion of PCA, the subsequent PCR-amplified product mixture still contains undesired shorter DNA fragments that hinder cloning efforts. To avoid tedious gel purification, an automated two-column purification has been developed and used in conjunction with rapid PCA. The system enables fast synthesis and isolation of the full-length DNA of interest, important for facile cloning of desired DNA fragments. During the PCR amplification step, forward and reverse primers tagged with iminobiotin and bromodeoxyuridine labels, respectively, were used. The automated purification was then performed on the PCR mixture using two affinity/immunocapture columns in series to isolate only the desired full-length product. The procedure has been applied to the pUC19 β-lactamase gene (929 bp). Follow-up PCR of the purified product, cloning, and sequencing demonstrated the technique's effectiveness in obtaining the pure full-length gene. The purification has also been performed on other synthesized genes, indicating its utility as a general approach.

Introduction

Synthesis of genes is of growing importance, especially for moderate to long sequences with biological functionality. Automated phosphoramidite synthesizers routinely create short (~15–100 bp) nucleotide sequences, but efficiencies prohibit feasible construction of longer sequences. Integrated DNA Technologies (Coralville, IA) commercially offers "ultramers" (synthetic oligonucleotides up to 200 bp in length) (1). A variety of techniques have been utilized, such as ligation and polymerase chain assembly that essentially "stitch" short oligonucleotide sequences together to form longer DNA sequences. Despite these techniques, gene synthesis of longer sequences remains rather laborious and error-prone (2).

The polymerase chain assembly (PCA) method to create longer synthetic genes from overlapping short oligonucleotides was first described by Stemmer et al. (3). Overlapping oligonucleotides are subjected to PCR thermocycling and polymerase extension to form the gene of interest. Stemmer et al. (3) assembled a 1.1 kb β-lactamase encoding gene and a 2.7 kb plasmid from short 40mer oligonucleotides. Many other researchers have since conducted PCA or variations thereof and in combination with other synthesis techniques to successfully construct longer genes (4–7). Recently, Gibson et al. (8) synthesized a 582,970 bp *Mycoplasma genitalium* genome using recombination techniques from 5–7 kb overlapping "cassettes" assembled from ~ 10^4 oligonucleotides of ~ 50 bp in length each.

Mamedov et al. (2) used rapid PCA to construct the endothelial protein-C receptor and thrombin receptor genes. The importance of rapid PCA lies in the minimization of errors produced in the assembly process, allowing for high fidelity and functional synthetic genes to be constructed in minutes. This technique is used for our synthesis of β-lactamase and h-TM; for a more detailed discussion on the technique, please refer to Mamedov et al. (2).

Despite advances in PCA, there remains a need for fast and reproducible methods to automatically purify the synthesized genes. During the PCA process, only a few copies of the full-length gene are constructed against a large background of shorter oligonucleotides. Traditionally, PCA is followed by primer-mediated PCR amplification to selectively amplify the full-length product. However, undesired oligonucleotides from the assembly are still significantly present in the PCR product, and some are linearly amplified. The resulting smear of products typically requires gel purification to isolate the desired product. However, band cutting is time-consuming and difficult, especially if the DNA bands are of similar sizes. For the synthesis of longer DNA, multiple DNA constructs need to be assembled, purified, and ligated. Manual separation of tens or even hundreds of each DNA constructs (each with different lengths) is slow and thus expensive.

Affinity chromatography, especially immunoaffinity chromatography, has grown considerably in use over the past several decades. The first application of affinity chromatography is commonly attributed to Starkenstein (9) while the first antibody-antigen immunocapture separations were developed in

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the 1930's (10,11). In modern times, a plethora of affinity-based systems are commercially available.

Incorporating labels into DNA is an attractive method for chromatographic purification. One commonly used interaction is the sepharose–biotin (or biotin analog) system (12–14). Additionally, the thymidine analog bromodeoxyuridine (BrdU) antibody-antigen systems also find common use in immunoaffinity separations, especially for *in vivo* labeling (15–19). However, to our knowledge, no one has attempted to use two separate DNA labels for serial purification of genes.

To circumvent gel purification, an automated purification technique has been developed that utilizes two affinity separations in series. During the PCR amplification step of PCA synthesized product, the forward and reverse primers have Iminobiotin and BrdU labels attached to their 5' ends, respectively. The amplified product is then subjected to a tandem affinity/immunocapture system. The product mixture is partially purified by an anti-BrdU Pab column, and the eluted intermediate product is purified further by a streptavidin affinity column to yield only the full-length product in the final effluent. A software interface allows the user to program the sequencing of pumps and valves to automatically perform the protocol. See Figure 1 for a schematic of the process.

The novelty of the approach detailed in this paper is in the utilization of labels on both the 5' and the 3' end of the assembled dsDNA fragment to allow a facile separation from shorter or incompletely assembled DNA fragments in a subsequent serial purification step. Labeling on both the 5' and 3' DNA ends enables isolation of the gene of interest that would otherwise be impossible by widely used single label approaches. The high level of discrimination of the full-length gene from a complex background facilitates successful cloning with minimal screening requirements. This becomes exceedingly important if one is conducting gene synthesis on larger scales, as multiple genes can be isolated and cloned facilely.

Materials and Methods

Oligonucleotide design and synthesis

Oligonucleotides spanning the template coding the pUC19 β-lactamase gene (929 bp) were designed from the cDNA sequence (GenBank accession no. L09137). The Gene2Oligo computer program (20) was used to optimize melting temperatures among the oligonucleotides. Fifty-two oligos with an average length of 37 nucleotides each at 50 µM in nuclease-free water were obtained from Integrated DNA Technologies (Coralville, IA). The forward Iminobiotin-labeled primer and reverse BrdU-labeled primer (both labeled on 5' ends) were obtained from IBA GmbH (Göttingen, Germany) while regular primers were obtained for Integrated DNA Technologies.

PCA and PCR of the pUC19 β-lactamase gene

PCA and PCR were performed in the PCRJet thermocycler (Megabase Research Products, Lincoln, NE) in accordance with the procedure of Mamedov et al. (2). The 25-µL assembly mixture contained 0.1 µM each oligonucleotide, 200 µM of each dNTP, 4 mM $MgSO_4$, 400 µg per mL non-acetylated BSA, and 0.5 unit KOD hot-start polymerase in 1x manufacturer's buffer obtained from Novagen (Madison, WI). PCA was conducted under the following conditions: 30 s hot start at 94°C, followed by 30 cycles of 94°C for 2 s, 56°C for 10 s, and 72°C for 10 s, and a final extension at 72°C for 25 s.

The PCA product was subsequently PCR amplified using the following primers (regular and labeled primers identical in sequence): 5-GTAAACTTGGTCTGACAGTTAC-3 (forward) and 5-TGAGACAATAACCCTGATAAATG-3 (reverse). Each 25-µL PCR reaction mixture contained 0.5 µL PCA product, 200 µM of each dNTP, 4mM $MgSO_4$, 400 µg/mL non-acetylated BSA, 0.7 µM each primer, and 0.5 unit KOD hot-start polymerase in 1x manufacturer's buffer. PCR amplification was conducted under the following conditions: 30 s hot start at 94°C, followed by 30 cycles of 94°C for 2 s, 55°C for 3 s, and 72°C for 10 s, and a final extension at 72°C for 25 s.

Column chromatography and manual purification

Polyclonal antibody against BrdU (anti-BrdU Pab) was purchased from Immunology Consultants Laboratory (Newberg, OR). Activated NHS-Sepharose resin was purchased from Sigma Chemical Company (St. Louis, MO). The anti-BrdU-Pab was coupled to the NHS-Sepharose resin per the protocol supplied by the manufacturer. Briefly, the resin as supplied was drained and washed with 1 M ice-cold HCl and decanted. The resin was subsequently washed with ~ 10 column volumes of 0.1 M sodium bicarbonate, 0.5 M NaCl, pH 8.0 buffer (ligand coupling buffer) prior to

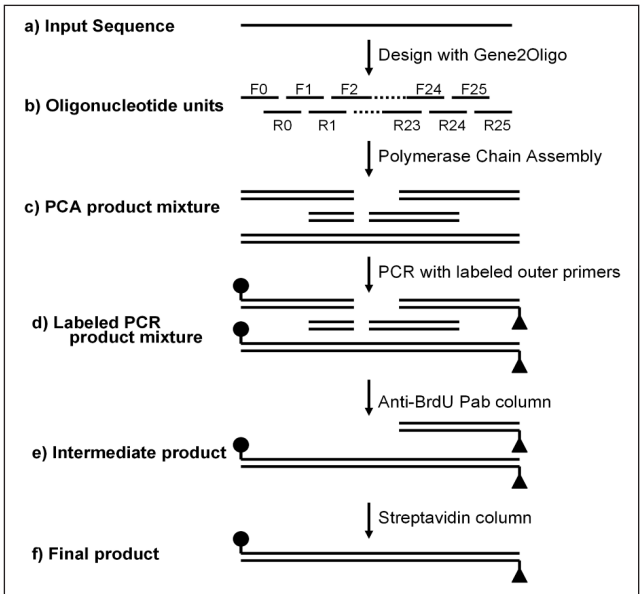

Figure 1. Schematic of the synthesis and purification protocol for the puc19 β-lactamase gene. 52 overlapping oligonucleotidedes were designed with the Gene2Oligo software (A). Oligonucleotides are mixed in equimolar concentrations and subjected to polymerase chain assembly (B). An aliquot of the PCA product is subjected to PCR amplification using labeled outer primers. The triangle and circle depict the BrdU and Iminobiotin labels, respectively (C). The resulting PCR product mixture is purified by an anti-BrdU Pab column (D). The intermediate elution product (E) is further purified by streptavidin chromatography to yield the isolated full-length product (F). An optional follow-up PCR amplification with regular primers can be performed on the final product.

antibody coupling. The target antibody was dissolved in 0.1 M sodium bicarbonate, 0.5 M NaCl, pH 8.0 buffer (ligand coupling buffer), mixed with decanted resin at a volume ratio of 3:1, and placed on an end-to-end rotator in a cold room (4°C). Upon completion of the antibody coupling step, the supernatant was drained, and excess reactive groups on the resin were blocked with 1 M ethanolamine, pH 8.0 for 1 h at room temperature. Upon completion of the blocking step, the resin was sequentially washed with 0.1 M sodium bicarbonate, 0.05 M Tris-HCl, 0.5 M NaCl, pH 8.0 and 10 mM Tris-HCI, 50 mM NaCI, pH 7.4. Unless used immediately, the antibody coupled gel was stored at a neutral pH in the presence of 0.05% NaN_3 at 2°C to 8°C.

PCR product with the BrdU tag was adjusted to the composition of the binding buffer of the anti-BrdU Pab column (Solution A: 10 mM Tris-HCI, 50 mM NaCI, pH 7.4) by mixing 100 µL of the PCR product with 100 µL of 20 mM Tris-HCI, 10 mM NaCI, pH 7.4 and was incubated with 250 µL of NHS-Sepharose-anti-BrdU resin for 6 h at 4°C on a end-to-end rotator. Upon completion of the binding step, the resin was allowed to settle, and the supernatant was pipetted out. Subsequently, the resin was washed thrice with two column volumes of Solution A per wash, and supernatant was removed at the end of each wash step. The captured PCR product was eluted with the incubation of the drained resin at the end of the wash step with 500 µL of elution buffer (Solution B: 20 mM Tris-HCI 500 mM NaCI, pH 7.4) for 6 h at 4°C on a end-to-end rotator. Eluted PCR product in the supernatant was analyzed by 1% agarose gel electrophoresis.

PCR product obtained with the Iminobiotin tag was adjusted to the composition of the binding buffer for the streptavidin sepharose column (Solution C: 50 mM ammonium carbonate, 0.5 M NaCI, pH 10.0) by mixing the Iminobiotin tagged PCR product with Solution C at a 1:3 ratio by volume and further loaded onto a streptavidin sepharose column that was obtained commercially and packed into disposable columns that operate under gravity. After washing with 10 column volumes of solution C, the captured PCR product was eluted with elution buffer (Solution D: 50 mM ammonium acetate, 0.5 M NaCI, pH 4.0). Eluted PCR product was analyzed by 1% agarose gel electrophoresis.

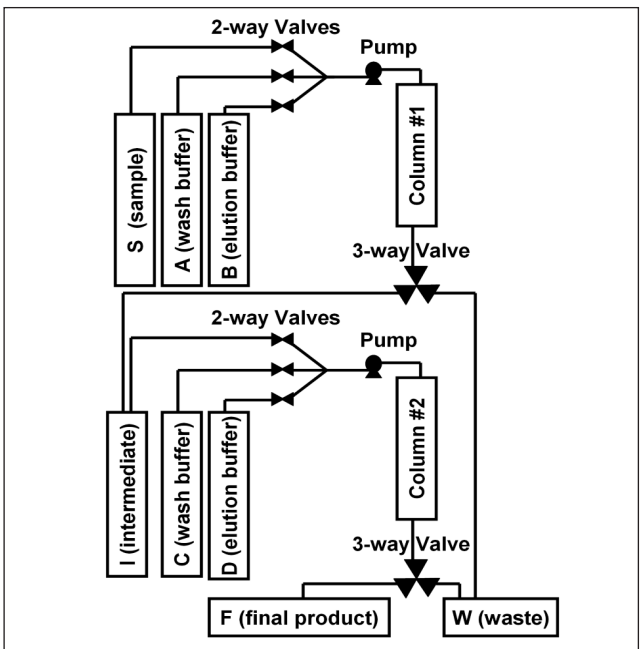

Figure 2. Schematic of the affinity/immunocapture workstation

The serial separation using both columns was run in the prototype workstation. Because of the compatibility of buffer chemistry, the anti-BrdU Pab column was utilized first, followed by the streptavidin column. Several key differences arose in the continuous flow tandem separation vs. the manual batch separation. First, optimal binding conditions of the feed mix to the anti-BrdU column were found to occur when the PCR product was mixed with sterile water at a 1:6 ratio by volume. Second, pH adjustment of the intermediate product (at anti-BrdU Pab elution buffer composition) was found to be most successful when diluting at a 1:3 ratio by volume with 1× streptavidin binding buffer (solution C).

Affinity/immunocapture system

The workstation components were mounted on a vertical platform; a schematic of the workstation is shown in Figure 2. The main components are described. Pumps: There are two pumps shown, one pump for each affinity column. The displacement diaphragm pumps (model # 120SP2410-4TE, Bio-Chem Valve Inc., Boonton, NJ) are factory-calibrated to 10 µL per pump stroke at a rate of up to 2 Hz.

2-way valves: There are six 2-way normally closed valves. These micro-isolation valves (model # 038T2S24-32-4, Bio-Chem Valve Inc.) provide a compact method to direct fluid flow within the workstation.

3-way valves: There are two 3-way isolation valves in the affinity workstation (model # 075T3MP42-32, Bio-Chem Valve Inc.). Each 3-way valve selects the direction for the fluid to flow downstream of the affinity columns, either directed towards the next column or towards a waste container.

Tubing and fittings: The pumps and valves are connected with chemically inert PTFE tubing (model # PTFE1030, Western Analytical Products, Wildomar, CA). This replaceable and sterilizable tubing has a 0.030" inner diameter and 1/16" outer diameter. Various PTFE and PEEK fittings and connectors were obtained to connect the workstation components (Western Analytical Products, Wildomar, CA).

Reagent vessels: Several reagent vessels are used in the workstation for storage of buffer solutions and waste collection. These reagent vessels are currently 50 mL centrifuge tubes (model # 2553, CLPdirect, San Diego, CA). The selection of vessel is a matter of convenience, and any other suitable container may be used.

Affinity/Immunocapture columns: A 1 mL HiTrap Streptavidin HP column (part # 17-5112-01, GE Healthcare, Piscataway, NJ) was used for the Iminobiotin capture. A custom anti-BrdU Pab was prepared and packed in a Tri-corn 5/20 glass column (part # 28-4064-08, GE Healthcare).

A custom printed circuit board (MIS Engineering, Lincoln, NE) received signals from a KPCI-3107 data acquisition board (Keithley Instruments, Inc., Cleveland, OH). A 24VDC power supply (model #MAP55-1024, Power-One, Camarillo, CA) was used to power the system. A software interface was developed in-house that allowed the user to program the protocol steps.

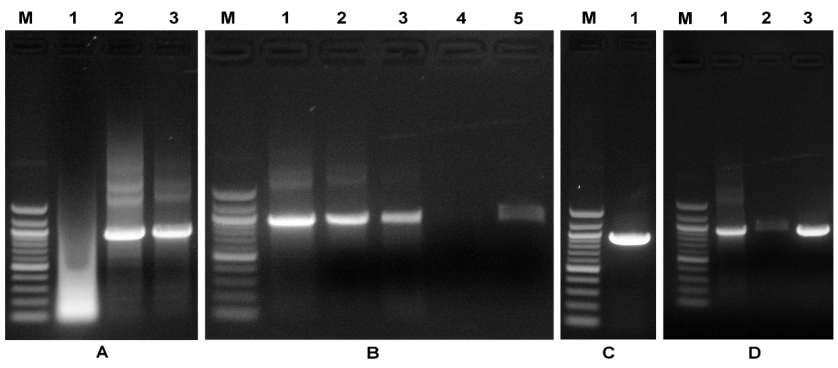

Figure 3. PCA, PCR, and Affinity Purification of the pUC19 β-lactamase Gene (929 bp).

PCA and PCR products. Lanes (M) 100 bp DNA ladder (NEbiolabs); lane 1: PCA product (8 µL); lane 2: PCR product with unlabeled primers (8 µL); lane 3: PCR product with labeled primers (8 µL) (A).

Affinity Separation. Lanes (M) 100bp DNA ladder (NEbiolabs); lane 1: 929 bp labeled PCR product (8 µL); lane 2: wash sample from BrdU column (30 µL); lane 3: intermediate elution product from BrdU column (30 µL); lane 4: wash sample from streptavidin column (30 µL); lane 5: final elution product from streptavidin column (30 µL) (B).

PCR of Affinity Purification Product. Lanes (M) 100bp DNA ladder (NEbiolabs); lane 1: 929 bp PCR product (8 µL) of the purified product from Figure 3B, lane 5 (C).

Fully Automated Affinity Separation. Lanes (M) 100 bp DNA ladder (NEbiolabs); lane 1: PCR product with labeled primers (sample S, 8 µL); lane 2: Final Elution Product (30 µL); lane 3: 929 bp PCR of the Affinity Product (8 µL) (D).

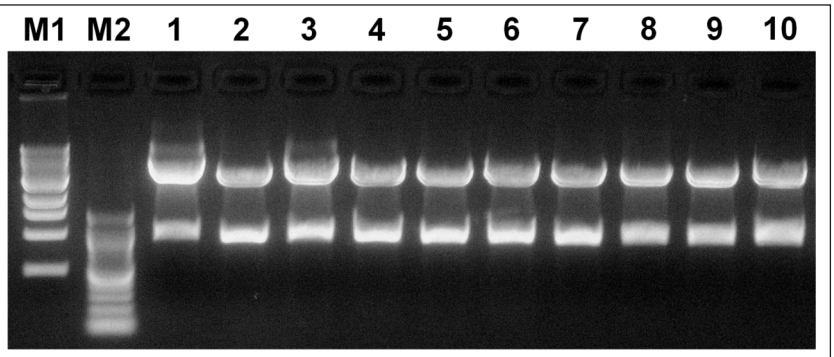

Figure 4. EcoR1 restriction enzyme digest of 10 clones. Lane M1: 1 kb DNA ladder (NEbiolabs); Lane M2: 100bp DNA ladder (NEbiolabs); lane 1–10: EcoR1 digest of 10 clones (10 µL each).

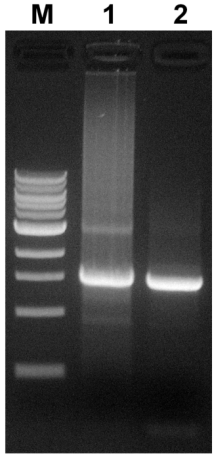

Figure 5. PCR products of the h-TM gene (1548 bp). Lane M: 1 kb DNA ladder (NEbiolabs); lane 1: PCR of the PCA product with labeled primers (8 µL); lane 2: PCR of the Affinity Workstation Product with regular primers (8 µL).

Automated purification protocol

The immunocapture workstation was initialized by priming lines with the respective solutions. The intermediate vessel was preloaded with 3 mL of 1× binding buffer (solution C), and the sample vessel was loaded with 200 µL of labeled PCR product mixture and 1200 µL sterile distilled water. Pumping was set at 1 Hz for an approximate flow rate of 0.6 mL/min. The software was programmed to run the following sequence: (*i*) Equilibriate columns with respective binding buffers; (*ii*) Load in sample mixture (S) to anti-BrdU Pab column; (*iii*) Wash BrdU column with binding buffer (solution A); (*iv*) Elute intermediate product from BrdU column with elution buffer (solution B); (*v*) Load intermediate mix (solution I) to streptavidin column; (*vi*) Wash streptavidin column with binding buffer (solution C); (*vii*) Elute final product from streptavidin column with elution buffer (solution D).

Cloning and sequencing

The purified product was ligated to TOPO vector using Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA). The ligated plasmid was transformed into competent cells of *E. coli* strain TOP-10 (Invitrogen), and 10 positive clones were selected. Purified plasmids from selected clones were sequenced at the University of Nebraska-Lincoln sequencing facility (CEQ8000 DNA Sequencer) using vector and gene specific primers.

Results and Discussion

The assembly product and PCR amplification (with both regular and labeled primers) of the assembly product is shown in Figure 3A. The success of the PCA is confirmed by the presence of the correct size full-length bands from the PCR amplifications. Note that a significant smear is present in the PCR steps due to a complex mixture of (i) carryover and linear amplification of short assembly oligonucleotides and (ii) duplex formation of different length single-stranded DNA with complementary sequence regions.

Samples from wash/elution fractions of the automated immunocapture protocol are shown in Figure 3B. As expected, the intermediate elution product (Figure 3B, lane 3) from the anti-BrdU Pab column has both the full-length product present along with undesired products that possess the BrdU label but not the Iminobiotin label. Subsequent purification of this intermediate through the streptavidin column results in only the full-length product with both labels in the elution fraction. The absence of visible bands in the streptavidin wash step (Figure 3B, lane 4) is attributed to the high dilution of the intermediate product. The slight upward shift of the final product (Figure 3B, lane 5) is due to the low pH of the elution buffer. The final elution

product was confirmed by subsequent PCR (Figure 3C). The eluted product and its PCR amplification of the automated procedure are shown in Figure 3D.

The EcoR1 digests of ten clones obtained from the purified product are shown in Figure 4. The gel indicates that the full-length gene insert was present in all clones. The presence of the full-length insert in all ten clones was confirmed by sequencing.

The procedure has also been used to isolate other synthesized genes. As an example, the human thromobomodulin gene (h-TM, 1548bp) was assembled as previously described by Mamedov et al. (2). The subsequent labeled PCR (of PCA product) and resulting PCR of the purified product is shown in Figure 5. Despite the differences in gene sizes, the chromatographic behavior was remarkably consistent among tested genes (results not shown). This indicates that the strategy can be used as a general technique for rapid synthesis and purification of multiple genes.

The rapid two-step PCA/PCR protocol of Mamedov et al. (2) was used to successfully construct the desired full-length product and amplify with labeled primers. Even though PCR amplification generated substantial full-length product, a background of undesirable products remained. Rather than conducting manual gel purification, a simple automated affinity workstation was employed, resulting in successful isolation of the desired full-length β-lactamase gene. The purification was verified by PCR amplification of the eluted product, cloning, and sequencing.

There are several benefits of the automated purification over manual gel purification. First, the procedure is faster than gel purification (< 30 min compared to ~1 h). Second, one need not be concerned with introducing synthetic DNA errors due to UV damage as in gel purification as any aliquots to gels need not be directly used for cloning. Additionally, the automation of this step reduces the labor burden thereby increasing the throughput potential for multiple gene synthesis by PCA.

Most importantly, the automated purification is more effective than gel purification in isolating the desired full-length gene. This improves cloning efforts as it is very likely that positive clones will possess the full-length insert and thereby reduce the amount of screening needed. This is supported by digest results of all ten clones in Figure 4. The rapid PCA procedure utilized minimizes gene synthesis errors, while the purification procedure maximizes cloning insertion of the full-length product. When combined, the end result is a procedure that minimizes the cloning and screening requirements to obtain synthetic genes.

Acknowledgements

This work was supported in part by funds from the NIH (1 R21 RR022860-01).

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Manuscript received November 10, 2008;
revision received January 12, 2009.