Research Article

Making *Taq* DNA polymerase in the undergraduate biology laboratory

Philip Ferralli, John Duick Egan, and Floyd Lester Erickson

Department of Biological Sciences, Salisbury University, Salisbury, MD 21801

Abstract. The polymerase chain reaction (PCR) is an important technique for biology students to learn. PCR utilizes DNA polymerases isolated from archaea or bacteria, like *Thermus aquaticus* (*Taq*), to amplify target DNA sequences. In this paper we describe lab activities where students clone the gene for, express, and purify *Taq* DNA polymerase and assay for its activity. These lab activities employ plasmids containing multiple components of the *lac* operon thereby giving students practical experience with a genetic regulatory system they learn about in the classroom. *Taq* DNA polymerase purification simply involves cell lysis, a heat incubation step, and centrifugation, with the resulting supernatant containing highly pure and active *Taq* DNA polymerase

Introduction

PCR was invented by Kary Mullis of the Cetus Corporation in 1983 and is a powerful method for the rapid amplification of target nucleic acid sequences (Mullis, et al., 1986). PCR is useful in gene cloning, DNA sequencing, gene expression analysis, DNA fingerprinting, and the detection of infectious and genetic disease disorders (Lo and Chan, 2006). The amplification process involves repeated cycles of heat denaturation of a DNA template containing the target sequence, annealing of opposing primers to the complementary DNA strands, and extension of the annealed primers with DNA polymerase and free deoxynucleotides. Multiple PCR cycles result in the exponential amplification of the nucleotide sequence between the flanking primers.

This original PCR technique was slow and labor-intensive because fresh DNA polymerase had to be added after every heat denaturation step. An important modification of the original PCR technique was the substitution of *Taq* DNA polymerase in place of the Klenow fragment of *Escherichia coli* (*E. coli*) DNA polymerase I (Saiki, et al., 1988). The PCR technique using *Taq* DNA polymerase was patented by Cetus Corporation in 1989 and the patent rights were later sold to the pharmaceutical company Hoffmann-La Roche for $300 million. In 1989 *Science* magazine named *Taq* DNA polymerase the "Molecule of the Year", and in 1993, Kary Mullis was awarded the Nobel Prize in Chemistry for developing PCR. Currently the market for *Taq* DNA polymerase is in the hundreds of millions of dollars per year.

Correspondence to: Floyd Lester Erickson, Department of Biological Sciences, Salisbury University, Salisbury, MD 21801; phone: (410) 543–6940; fax: (410) 543–6433; e-mail: flerickson@salisbury.edu

Bios 78(2) 69–74, 2007

Taq DNA polymerase was first isolated in 1976 (Chien, et al., 1976) from *Thermus aquaticus* isolated from a hot spring in Yellowstone National Park (Brock and Freeze, 1969). Because the purification of *Taq* DNA polymerase from the native host results in low yields, the gene encoding this enzyme was cloned and overexpressed in *E. coli* (Lawyer, et al., 1989; Engelke et al., 1990).

In this paper, we describe the cloning of the *Taq* DNA polymerase gene, recombinant protein expression, and purification schemes that we use as laboratory activities in an undergraduate genetics course. Our protocols are adapted from published methods for the rapid purification of recombinant *Taq* DNA polymerase (Pluthero, 1993; Desai and Pfaffle, 1995), with modifications that make them more suitable for the time course of a typical undergraduate laboratory.

Lab Activities

Amplification of the *Taq DNA polymerase* gene

Thermus aquaticus (ATCC number 31558) are grown (3–5 days) to saturation at 70° C in growth media (0.1% yeast extract, 0.1% tryptone). Students isolate genomic DNA from 10 ml of culture using a genomic DNA protocol developed for yeast (Hoffman and Winston, 1987), followed by ethanol precipitation, resuspension in 200 µl Tris/EDTA buffer, and quantitation using UV spectroscopy.

Students use the *Taq* genomic DNA to amplify the open-reading-frame of the *Taq DNA polymerase I* gene (Gene Bank accession J04639) using PCR and gene-specific DNA primers designed by Engleke *et al*. (Table 1). The upstream primer includes an *Eco*RI restriction site, and the downstream primer includes a *Bgl*II restriction site. PCR is performed in a 25 µl volume containing 12.5 µl of 2× PCR master mix (Promega M750B), 10 pmol each primer, and 250 ng of *Taq* DNA. PCR is performed in a thermocycler programmed at 94° C for 2 minutes followed by 35 cycles of 94° C for 1 minute, 55° C for 1 minute, 72° C for 3 minutes. A portion of the PCR is analyzed by gel electrophoresis to confirm the presence of a 2500 base-pair amplicon (Figure 1A).

Cloning of the *Taq* DNA polymerase gene

Students clone the *Taq* DNA polymerase gene sequences into either the expression plasmid pTTQ18, which results in a functional clone for gene expression, or the cloning plasmid pGEM T-easy, which is procedurally simpler but results in a clone not useful for gene expression. During the first semester these lab activities were performed, students used pTTQ18, but we now have them use pGEM T-easy.

To clone the *Taq* DNA polymerase gene using pTTQ18, 16 µl of the amplicon is digested with 10 units each of the restriction enzymes *Eco*RI and *Bgl*II in 20 µl of buffer D (Promega). One µg of pTTQ18 (Figure 1B) is digested for 1 hour with 10 units each of the restriction enzymes *Eco*RI and *Bam*HI (note that *Bgl*II sticky ends can ligate into *Bam*HI sticky ends) in 20 µl of

Table 1. Primers and buffers. The underlined sequences within the primers are the added restriction enzyme digestion sites.

Buffer A	50 mM Tris-HCl pH 7.9, 50 mM dextrose, 1mM EDTA
Buffer B	10 mM Tris-HCl pH 7.9, 50 mM KCl, 0.5% Tween 20 detergent, 0.5% Nonidet P40 detergent
Storage Buffer	50 mM Tris-HCl pH 7.9, 100 mM NaCl, 0.5 mM DTT, 1% triton X-100 detergent, and 50% glycerol
Upstream primer	5'-CACGAATTCGGGGATGCTGCCCCTCTTTGAGCCCAAG-3'
Downstream primer	5'-GTGAGATCTATCACTCCTTGGCGGAGAGCCAGTC-3'

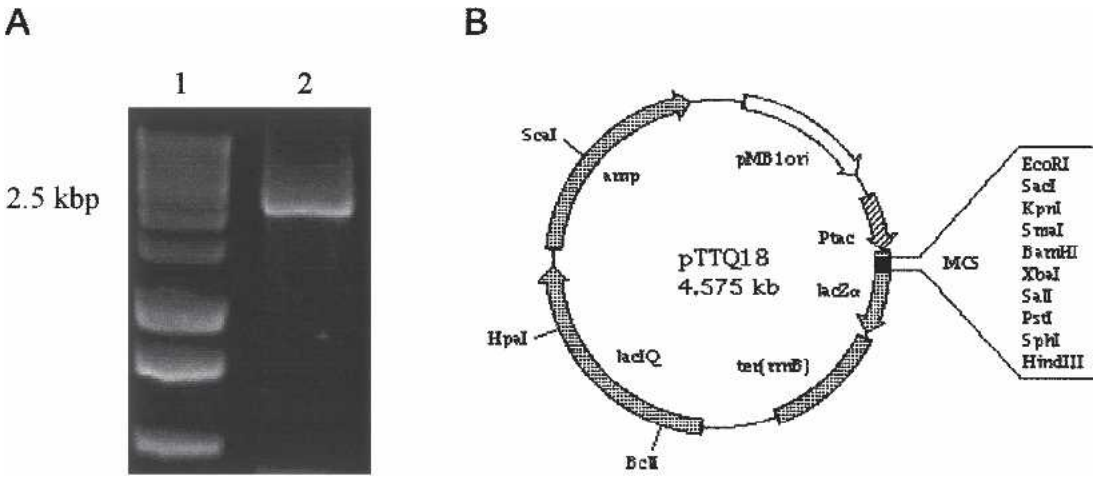

Figure 1. *Taq* DNA polymerase amplicon and pTTQ18 plasmid map. A) Eight µl of the PCR was mixed with 2 µl of 10X loading dye and electrophoresed through a 1% agarose gel in tris-acetate-EDTA buffer and ethidium bromide (0.1 µg/ml). The gel was visualized using a UV light box and photographed using a digital camera. Lane 1 is DNA size standards. Lane 2 is PCR of *Taq* DNA polymerase gene. B) Map of plasmid pTTQ18 used to clone and express the *Taq* DNA polymerase gene.

multicore buffer (Promega). The DNA digestions are electrophoresed through a 1% agarose gel, and the DNA bands are visualized using ethidium bromide and UV light. The amplicon and plasmid bands are excised from the gel, the two gel fragments combined, and the DNA purified using the QIAquick gel extraction kit (Qiagen, Inc.) following the manufacturer's instructions. Twelve µl of the purified DNA mixture, 1.5 µl of ligation buffer, and 1.5 µl T4 DNA ligase (New England Biolabs) are combined and the ligation reaction is incubated overnight at 15° C.

The above cloning activity can be time consuming and the multiple steps involved often leads to student error. For these reasons, we have found it easier if students clone the amplicon by ligating directly into the pGEM T-Easy cloning vector (Promega Corp.) using T/A cloning. The T/A cloning method is quicker than using restriction enzymes to clone because it takes advantage of the single, 3′-deoxyadenosine overhang added to ends of the PCR product by the terminal transferase activity of *Taq* DNA polymerase. This makes it possible to ligate PCR products directly into commercially-prepared, linearized cloning vectors, like pGEM T-Easy, that contain single, 3′-deoxythymidine overhangs. Students ligate agarose gel-purified amplicons into pGEM T-easy plasmid following the Promega ligation protocol.

The completed ligation reactions, involving either pTTQ18 or pGEM T-easy, are used to transform *E. coli*, and colonies containing recombinant plasmid are identified by ampicillin resistance and blue-white screening. Five µl of ligation reaction is incubated with 50 µl chemically-competent *E. coli* cells (JM109) on ice for 30 minutes. The cells are heat shocked at 42° C for 50 seconds and then placed on ice for 2 minutes. Five hundred µl of LB medium is added to the cells, followed by 30 minute incubation with shaking at 37° C. 100 µl of cells are then plated on LB agar plates containing 100 µg/ml ampicillin. For transformations involving pGEM T-easy, 40 µl of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal; 20 mg/ml stock in dimethyl formamide) and 40 µl of isopropyl-beta-D-thiogalactopyranoside (IPTG; 24 mg/ml stock in water) are also added to the plates prior to plating the bacteria. After an overnight incubation at 37° C, ampicillin-resistant colonies containing recombinant pGEM T-easy plasmids are identified using blue/white screening. Cloning into pGEM T-Easy disrupts the *lacZ* gene on the plasmid, resulting in cells that harbor recom-

binant plasmids appearing white on X-gal-containing agar plates; non-recombinant plasmids produce blue colonies due to the cleavage of X-gal by the *lacZ* gene product, β-galactosidase.

Because pGEM T-easy plasmids are not useful for protein expression, we do not have students further characterize these recombinant plasmids beyond counting the blue and white colonies on the transformation plates. Recombinant pTTQ18 plasmids are identified using a Qiagen mini-prep kit and restriction digestion analysis to confirm the presence of the amplicon. An *E. coli* strain containing a recombinant pTTQ18/*Taq DNA polymerase I* plasmid has been designated Fp251, and although the cloned gene has not been sequenced to check for PCR-induced mutations, functional *Taq* DNA polymerase is produced using this plasmid (see below). All students use this strain for expression and purification of *Taq* DNA polymerase.

Expression and purification of *Taq* DNA polymerase

The purification of *Taq* DNA polymerase from *E. coli* is relatively simple because of high expression levels and the thermostable nature of the enzyme, which allows a "heat-cut" to denature contaminating host proteins. To express *Taq* DNA polymerase, the instructor grows *E. coli* strain Fp251 to saturation in 5 ml of LB broth with ampicillin (100 μg/ml) at 37° C in a shaker. This culture is used to inoculate 200 ml of LB broth with ampicillin in a 1 liter flask. The culture is grown at 37° C in a shaker at 225 rpm to an A_{600} between 0.5 and 1.0. Gene expression from the plasmid is induced by adding IPTG to 0.2 mM and the culture is grown for an additional 12–16 hours. At this time the culture can be placed on ice for a few hours if needed.

In the laboratory, each student pair harvests 30 ml of cells using centrifugation at 4300 rpm for 5 minutes. The supernatant is removed and the cell pellet is resuspended with 3 ml of buffer A (Table 1) containing 4 mg/ml lysozyme, and the cell suspension is incubated at room temperature for 10 minutes to promote cell lysis. An equal volume of buffer B (Table 1) is then added, followed by incubation in a 75° C water bath for 30 minutes with mixing by inversion every 5 minutes. The resulting lysate is transferred into a centrifuge tube and spun at 12,000 rpm for 10 minutes at 20° C in a high speed centrifuge. After centrifugation the supernatant fraction is transferred to a clean tube and mixed with an equal volume of storage buffer (Table 1) and stored at 4° C.

Students analyze 10 μl of the diluted supernatant fraction using SDS-polyacrylamide gel electrophoresis. As shown in Figure 2 a single protein species at the predicted size of *Taq* DNA polymerase (94 kD) is apparent (lane 2). No protein species are observed from an uninduced control culture where no IPTG was added (Figure 2, lane 1).

Assay for *Taq* DNA polymerase activity

To assay for *Taq* DNA polymerase activity, students perform PCR using the supernatant fraction as the DNA polymerase component. For

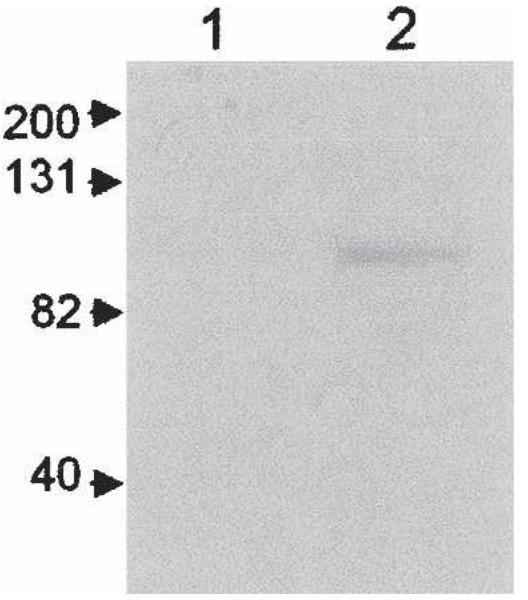

Figure 2. Analysis of *Taq* DNA polymerase purification from *E. coli* using SDS-PAGE and Coomassie blue staining. The positions and sizes (in kilodaltons) of the molecular weight markers are indicated on the left. Ten μl of the high-speed supernatant fraction was mixed with 10 μl of 2× loading buffer and electrophoresed through a 12% SDS-polyacrilamide gel (Bio-Rad) which was stained with coomassie blue. Lane 1 is from an uninduced Fp251 culture. Lane 2 is from an induced Fp251 culture.

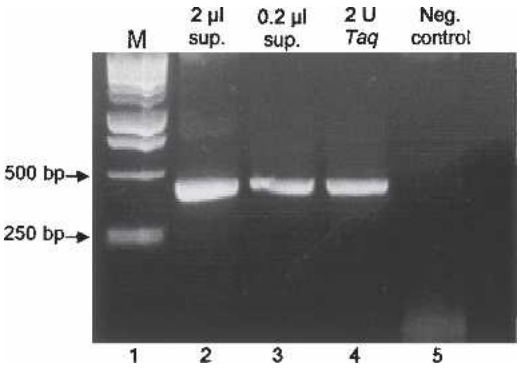

Figure 3. Agarose gel showing *Taq* DNA polymerase activity. Eight µl of the PCR was mixed with 2 µl of 10× loading dye and electrophoresed through a 1% agarose gel in Tris-acetate-EDTA buffer and ethidium bromide (0.1 µg/ml). The gel was visualized using a UV light box and photographed using a digital camera. Lane 1 is DNA size standards. Lane 2 used the purified *Taq* DNA polymerase. Lane 3 used a 1:10 dilution of the purified DNA polymerase. Lane 4 used 2 units of commercial *Taq* DNA polymerase (Sigma RedTaq). Lane 5 was a control PCR lacking template DNA, but used *Taq* DNA polymerase as in lane 2.

comparison, PCR is also performed using 2 units of a commercial *Taq* DNA polymerase preparation. A stock plasmid (pACT) and corresponding primers are used in the PCR assay that should result in the amplification of a 300 base-pair amplicon. Reactions contain 2.5 µl 10× PCR buffer with $MgCl_2$ (Promega), 0.3 mM dNTPs, 10 pmol each of forward and reverse primers, 0.1 µg template DNA, 2 µl (or 2 µl of 1/10 dilution) of *Taq* protein fraction, and water to 25 µl final volume. PCR is performed in a thermocycler programmed for 94° C for 2 minutes followed by 35 cycles of 94° C for 30 seconds, 55° C for 30 seconds, 72° C for 1 minute. Agarose gel electrophoresis shows that the expected 300 base-pair fragment is produced by both the student purified and commercial *Taq* DNA polymerase preparations (Figure 3, lanes 2–4). Based on the relative intensity of the amplicons in the stained gel, 2 µl of 1/10 diluted supernatant fraction (lane 3) is at least equivalent to 2 units of commercial *Taq* DNA polymerase (lane 2) indicating the student-prepared protein fraction has about 10 units/µl of DNA polymerase activity.

Additional comments

In this lab activity, each student pair produces 12 ml of a *Taq* DNA polymerase solution. Assuming a DNA polymerase activity of 10 units/µl, this means a lab of 20 students produces a little over 1 million units of enzyme. At the current cost of ~$0.50/unit for commercial *Taq* DNA polymerase preparations, a lab section of 20 students produces a half of million dollars worth of enzyme (students really enjoy doing this kind of math!). Because so much active enzyme is produced, the amount of starting cells could be reduced to 1.5 ml per student pair, which would allow for the purification process including the centrifugation step to be performed in a microcentrifuge tube.

In addition to learning about PCR, primer design, molecular cloning, protein analysis, and biotechnology, numerous components of the *lac* operon are used in these laboratory activities thereby giving students hands-on experience with a genetic system they learn about in the classroom. Blue/white screening involves β-galactosidase, the product of the *lacZ* gene. Plasmid pTTQ18 also contains the *lacI* gene encoding the lac repressor protein that binds to the operator element, *lacO*, within the transcriptional promoter used in pTTQ18. This makes gene expression dependent on the addition of IPTG, a nonhydrolyzable lactose mimic that binds to and inactivates the lac repressor.

In the U.S., the patents covering the PCR process including the production and use of *Taq* DNA polymerase have recently expired. These patent expirations provide an opportunity for many life science researchers and teachers to produce and use their own supply of *Taq* DNA polymerase. The protocols described in this paper produce a large quantity of pure and active enzyme that can be used in both teaching and research laboratories. When stored in 50% glycerol at −20° C, our *Taq* DNA polymerase preparations remain active for at least a year.

Acknowledgements. We thank Mark Holland and Adam Lowe for helpful comments on the manuscript and the Salisbury University students in Molecular Genetics (BIOL370) who helped test these protocols. Thanks to Paul Good and David R. Engelke for the pTTQ18 plasmid. This work was supported by USDA Higher Education Challenge Grant 2003-38411-13494 and

a Salisbury University Henson School of Science Undergraduate Research Grant.

More information about the laboratory activity can be found at www.salisbury.edu/biology/faculty/flerickson/FLElab.htm.

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Received 8 September 2006; accepted 19 February 2007.