



Amplifying Lambda DNA by Polymerase Chain Reaction (PCR)*

Amplification Reagents and Supplies FOR TEACHING PURPOSES ONLY

Instructor's Manual

(for manual or automated thermal cycling)

This kit was developed in cooperation with the DNA Learning Center of Cold Spring Harbor Laboratory. The experiment is adapted from *Laboratory DNA Science: An Introduction to Recombinant DNA Technology and Methods of Genome Analysis* by Mark V. Bloom, Greg A. Freyer, and David A. Micklos, copyright 1994, Cold Spring Harbor Laboratory

Upon receipt of the kit, store *Lambda* DNA, PCR mix and magnesium chloride solution in a refrigerator at *between 2° and 8°C* until use. Other materials may be stored at room temperature (approximately 25°C).

Individuals should use this kit only in accordance with prudent laboratory safety precautions and under the supervision of a person familiar with such precautions. **Use of this kit by unsupervised or improperly supervised individuals could result in serious injury.** This product is sold under distribution arrangements with the Perkin-Elmer Corporation.

* Polymerase chain reaction is covered by patents owned by Hoffmann-La Roche, Inc.

DNA AMPLIFICATION BY POLYMERASE CHAIN REACTION

Since its introduction in 1985, polymerase chain reaction (PCR) has become a powerful tool in molecular genetic analysis and has been cited in well over 7,000 scientific publications (as of 1993). PCR is a test tube system for DNA replication that allows a "target" DNA sequence to be selectively amplified, or enriched, several million-fold in just a few hours. Within a dividing cell, DNA replication involves a series of enzyme-mediated reactions, whose end result is a faithful copy of the entire genome. Within a test tube, PCR uses just one indispensable enzyme - DNA polymerase -- to amplify a specific fraction of the genome.

During cellular DNA replication, enzymes first unwind and denature the DNA double helix into single strands. Then, RNA polymerase synthesizes a short stretch of RNA complementary to one of the DNA strands at the start site of replication. This DNA/RNA heteroduplex acts as a "priming site" for the attachment of the DNA polymerase, which then produces the complementary DNA strand. During PCR, high temperature is used to separate the DNA molecule into single strands, and synthetic sequences of single-stranded DNA (20-30 nucleotides) serve as primers. Two different primer sequences are used to bracket the target region to be amplified. One primer is complementary to one DNA strand at the beginning of the target region; a second primer is complementary to a sequence on the opposite DNA strand at the end of target region.

To perform a PCR reaction, a small quantity of the target DNA is added to a test tube with a buffered solution containing DNA polymerase, short oligonucleotide primers, the four deoxynucleotide building blocks of DNA, and the cofactor $MgCl_2$. The PCR mixture is taken through replication cycles consisting of:

- 1 to several minutes at 94-96°C, during which the DNA is denatured into single strands,
- 1 to several minutes at 50-65°C, during which the primers hybridize or "anneal" (by way of hydrogen bonds) to their complementary sequences on either side of the target sequence, and
- 1 to several minutes at 72°C, during which the polymerase binds and extends a complementary DNA strand from each primer.

As amplification proceeds, the DNA sequence between the primers doubles after each cycle. Following thirty such cycles, a theoretical amplification factor of one billion is attained.

Two important innovations were responsible for automating PCR. First, a heat-stable DNA polymerase was isolated from the bacterium *Thermus aquaticus*, which inhabits hot springs. This enzyme, called the *Taq* polymerase, remains active, despite repeated heating during many cycles of amplification. Second, DNA thermal cyclers have been invented in which a computer controls the repetitive temperature changes required for PCR.

In this experiment, students use PCR to amplify a 1106 base-pair sequence from the bacteriophage *Lambda* genome. The fact that the target sequence represents a relatively large proportion of the total *Lambda* genome (48,502 basepairs) increases the efficiency of the reaction, so only two temperatures are required for each replication cycle. Primer extension occurs as the reaction heats from the annealing temperature (55°C) to the denaturation temperature (100°C). The temperature may be controlled automatically by a thermal cycler or

manually using two water baths. The number of cycles can be varied among several reactions to illustrate the time course of the amplification.

Following amplification, samples are loaded in 1% agarose gels, electrophoresed, and stained with ethidium bromide or methylene blue. The size of the expected amplification product can be verified by comparison to an appropriate DNA molecular weight standard.

MATERIALS

The amplification reagents and supplies in the DNA Amplification by Polymerase Chain Reaction (PCR) Kit are sufficient for 50 reactions. Prior knowledge of basic methods of agarose gel electrophoresis and staining of DNA is presumed. The materials are supplied for use with the exercise in this kit only. Roche Molecular Systems, Inc., Perkin-Elmer Corporation, and Carolina Biological Supply Company disclaim all responsibility for any other uses of these materials. The kit includes:

Vial <i>Lambda</i> DNA (500 μ l)	Dropper Vial Mineral Oil (5 ml)
Vial PCR Mix (1000 μ l)	50 PCR Tubes
Vial MgCl ₂ Solution (1000 μ l)	Instructor's Manual
Vial Molecular Weight Standard (100 μ l)	12 Student Guides

Upon receipt of the kit, store *Lambda* DNA, PCR mix, and MgCl₂ solution in a refrigerator between 2° and 8°C until use. Other materials may be stored at room temperature (approximately 25°C).

Equipment needed but not provided are: programmable DNA thermal cycler or 55°C and 100°C water baths, micropipettors (1-10/20 μ l and 10-100 μ l), electrophoresis chambers with casting trays and well-forming combs, electrophoresis power supplies, mid- or long-wavelength UV light source with protective screen or glasses (for ethidium bromide staining), white light source (for methylene blue staining), Polaroid "gun" camera (optional), microcentrifuge (optional), and microwave oven (optional).

Reagents and supplies needed but not provided are: electrophoresis buffer, electrophoresis grade agarose, loading dye, ethidium bromide or methylene blue staining solution, micropipet tips, 1.5 ml polypropylene test tubes (100), test tube racks, and permanent laboratory markers.

SCHEDULING

DNA amplification by PCR requires several different activities. Plan your time as follows:

Day	Time Needed	Activity
Lab Period 1	30 min 10 min 45 min	Pre-lab: Set Up Work Stations Set Up PCR Reactions Perform PCR Amplification
Lab Period 2	10 min 10 min 10 min 40 min 20 min 20 min 10 min	Pre-lab: Prepare 1% Agarose Solution Cast Agarose Gels Load DNA Samples into Gels Electrophorese Post-lab: Stain Gels Post-lab: Destain Gels Post-lab: Photograph Gels
Lab Period 3	30 min	Results and Discussion

PRE-LAB PREPARATION

Suggested Experimental Set Up

The Pre-Lab Preparation and Lab Procedure are written for an experiment configured to show a time course for DNA amplification. Each lab group prepares a master reaction from which four samples are drawn: three samples are amplified for differing numbers of cycles, while the fourth sample is an unamplified control. To conserve reagents, the unamplified control may be omitted.

For Lab Period 1

1. *Lambda* DNA, PCR mix, and MgCl₂ may collect in cap tops during shipping. To have full volume available for student use, it may be necessary to pool these reagents by spinning tubes briefly in a microcentrifuge or rapping tube sharply on lab bench.
2. Store *Lambda* DNA, PCR mix, and MgCl₂ solution in a beaker of cracked ice during the experiment.
3. For each student experiment, aliquot the following volumes of reagents in 1.5 ml test tubes:
 - 50 µl *Lambda* DNA (on ice)
 - 100 µl PCR mix (on ice)
 - 100 µl MgCl₂ (on ice)
4. Prepare student stations, each with the following materials:

Tube <i>Lambda</i> DNA (50 µl)	Test Tube Rack
Tube PCR mix (100 µl)	10-100 µl Micropipet
Tube MgCl ₂ (100 µl)	Micropipet Tips
1.5 ml Test Tube	Permanent Marker
4-0.5 ml PCR Tubes	Student Guide
5. Students share the following materials: mineral oil, thermal cycler or two water baths (55°C and 100°C), microcentrifuge (optional).
6. For manual thermal cycling: Set up one boiling water bath (100°C) and one 55°C water bath. Fashion a test tube rack of styrofoam or other floating material to accommodate all PCR tubes; attach a handle, so it can be easily moved between the two water baths during amplification. A simple boiling water bath can be made by using a beaker of water and a hot plate. Replace evaporated water as necessary during experiment, so that water level does not drop below the level of the PCR tubes.

For automated thermal cycling: Program thermal cycler:

96°C 1 minute

58°C 1 minute, 1 cycle

link to:

96°C 30 seconds

58°C 1 minute, 16 cycles

link to:

58°C 10 minutes

For Lab Period 2

1. Prepare sufficient 1% agarose solution for the number of gels needed to electrophorese all student PCR reactions, plus one DNA marker lane per gel.
2. Prepare student stations, each with the following materials:

4-PCR Reactions (from Lab Pd. 1)	1-10 μ l Micropipet
Molecular Weight Standard (20 μ l)	10-100 μ l Micropipet
Loading Dye	Micropipet Tips
4-1.5 ml Test Tubes	Test Tube Rack
3. Students share the following materials: 1% agarose solution, electrophoresis buffer, electrophoresis chambers with casting trays and well-forming combs, staining trays, and 1 μ g/ml ethidium bromide or 0.025% methylene blue staining solution.

FINE POINTS OF LAB PROCEDURE

Be alert to the following cautions when performing the experiment. Where appropriate, discuss fine points with students, and have them make annotations on their Student Guides.

Preparation of Reagents Supplied

Upon first use of the DNA Amplification by PCR Kit, remove tube of PCR Reaction Mix and carefully aliquot 100 μ l into clean 1.5 ml tubes. Ensure that tubes are capped tightly. Place PCR Reaction tubes in a rack not used for DNA preparation or amplified DNA handling. Store tubes separated from any source of DNA at 2° to 8°C. Note: All of the PCR Reaction Mix should be aliquoted at the same time to decrease the possibility of contamination through repeated opening of the PCR Reaction Mix tube.

Setting Up PCR Reactions

DNA thermal cycling should be started as soon as possible after preparing PCR reactions. Nonspecific priming may occur if PCR reactions sit too long without cycling.

Amplification

Note differences in temperatures and cycle number between manual and automated thermal cycling. *For manual thermal cycling:* be careful not to leave samples in boiling water bath longer than the recommended time. During a 20-second incubation, samples warm up to a denaturing temperature of approximately 96°C. However, a prolonged incubation brings the sample temperature up to boiling, which reduces or destroys the activity of the *Taq* polymerase. *For automated thermal cycling:* use a "step file" that waits until the programmed temperature is reached before activating the cycle timer. The amplification program used here refers to the DNA Thermal Cycler from Perkin-Elmer Corporation. Use of other equipment may require times and temperatures to be adjusted.

DNA Markers

It is a good idea to include, on each gel, DNA markers of known size, such as a *Hind*III digest of *Lambda* DNA. The 1106 base pair (bp) PCR product will electrophorese ahead of the *Lambda/Hind*III doublet (2322 bp and 2027 bp) and behind the 564 bp fragment. The DNA size markers also serve as a control to aid in trouble-shooting potential problems with amplification, electrophoresis, and staining/destaining. For example, a very faint PCR product (compared to the markers) may indicate insufficient amplification and can be increased by adding four or five cycles to the thermal cycling.

Ethidium Bromide Staining

The gel thickness needed to contain a 20 μ l sample requires a gel staining time of approximately 10 minutes in a fresh 1 μ g/ml ethidium bromide solution. To best visualize the change in amount of PCR product over the amplification time course, it is necessary to increase contrast between the stained PCR product and unbound ethidium bromide in the gel ("background") by destaining the gel for 10 or more minutes in tap water. **Wear rubber gloves, and observe prudent cleanup and disposal procedures when working with ethidium bromide.**

Methylene Blue Staining

Stain gel in 0.025% methylene blue solution for 20 to 30 min. Destain for 30 or more minutes in several changes of tap water. PCR product bands become increasingly distinct as gel destains, decreasing "background" of unbound methylene blue.

Viewing and Photographing Gels

Take care to view and photograph gels as soon as possible after appropriate destaining. Over time, PCR product disappears from stained bands as it slowly diffuses through the gel.

LAB PROCEDURE

Procedure A: Set Up PCR Reactions

1. Use permanent marker to label a 1.5 ml tube "Master Reaction." Use the table below as a checklist while adding reagents to Master Reaction Tube.

<i>Lambda</i> DNA	PCR Mix	MgCl ₂
45 µl	90 µl	90 µl

2. Use permanent marker to label (on tube caps) four 0.5 ml PCR tubes, according to the type of thermal cycling you will employ:

<i>Manual Thermal Cycling</i>	<i>Automated Thermal Cycling</i>
0 Cycles (Control)	0 Cycles (Control)
10 Cycles	9 Cycles
15 Cycles	13 Cycles
20 Cycles	17 Cycles

3. Add 50 µl reactants from the Master Reaction Tube to each of the four PCR Tubes labeled in Step 2.
4. Add one drop of mineral oil from its dropper bottle to each of the four PCR Tubes. *Take care not to touch dropper to PCR reactants or tube.*
5. Place the PCR tubes on ice. The 0-Cycles (Control) Tube will remain on ice during thermal cycling steps.
6. Proceed with Manual or Automated Thermal Cycling Procedure below.

Manual Thermal Cycling (20 Cycles Total)

7. Load 20-Cycle Tube(s) in test tube rack, and place rack in boiling water bath for 20 seconds. Then move test tube rack to 55°C water bath for 1 minute. This is one thermal cycle.
 8. After 5 cycles have been completed, add the 15-Cycle Tube(s) to the rack, and continue cycling.
 9. After 10 cycles have been completed, add the 10-Cycle Tube(s) to rack. Continue cycling for an additional 10 cycles.
 10. Following 20 amplification cycles, allow the samples to remain at 55°C for a further 10 minutes.
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Automated Thermal Cycling (17 Cycles Total): To Be Performed by Instructor

7. Load 17-Cycle Tube(s) into thermal cycler, and begin the program:
Program and start thermal cycler:
96°C 1 minute
58°C 1 minute, 1 cycle
link to:
96°C 30 seconds
58°C 1 minute, 16 cycles
link to:
58°C 10 minutes
 8. After 4 cycles have been completed, place the 13-Cycle Tube(s) into the thermal cycler. *Be careful not to touch the heating block!*
 9. After 8 cycles have been completed, place the 9-Cycle Tube(s) into the thermal cycler. The thermal cycler will complete an additional 9 cycles.
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Procedure B: Cast, Load, and Electrophorese 1% Agarose Gel(s)

1. Cast an agarose gel to a depth of approximately 8 mm.
2. Use permanent marker to label four 1.5 ml tubes:

<i>Manual Thermal Cycling</i>	<i>Automated Thermal Cycling</i>
0 Cycles (Control)	0 Cycles (Control)
10 Cycles	9 Cycles
15 Cycles	13 Cycles
20 Cycles	17 Cycles
3. Transfer 20 µl of each PCR sample into the appropriate 1.5 ml tube. *Take care not to transfer any of the mineral oil from the PCR tube. Submerge pipet tip through mineral oil layer to draw off sample, and use tissue to wipe off any mineral oil clinging to the outside of the pipet tip.*
4. Add 2 µl of loading dye to each 1.5 ml tube. Close each tube top, and mix by tapping tube bottom on lab bench, pipetting in and out, or pulsing in a microcentrifuge.
5. Add 20 µl of each PCR/loading dye sample into adjacent wells of a 1% agarose gel. Expel any air in tip before loading, and be careful not to punch tip of pipet through bottom of gel.
6. Add 2 µl loading dye to a 20 µl aliquot of DNA size markers, and load one 20 µl sample per gel.
7. Electrophorese at 100 volts for about 40 minutes. Adequate separation will have occurred when the bromophenol blue dye front has moved 40 to 50 mm from wells.
8. Turn off power supply, remove casting tray, and transfer gel to disposable staining tray. Take gel to controlled area for staining, viewing, and photographing.

RESULTS AND DISCUSSION

1. Examine a photograph of the stained gel. Orient the photograph with the samples wells at the top, and compare your results with the expected results:
 - a. The control (unamplified) lane should show no trace of DNA bands.
 - b. The amplified samples should each show a single DNA band of 1100 base pairs.
 - c. The intensity (brightness) of the PCR fragments should increase with the cycle number.
 - d. A faint, diffuse band may be visible near the bottom of the gel. This so-called "primer dimer" is not amplified *Lambda* DNA, but is an artifact of the amplification process that results from primers amplifying themselves.
2. DNA size markers can be used to verify the size of the PCR product. First, the size of the PCR product can be estimated by comparing its position on the gel with those of the DNA size markers. However, a more accurate value can be obtained by graphing the function that determines the migration of linear DNA fragments in an electrophoretic field:

$$D = \frac{1}{\log MW}$$

where D equals distance migrated and MW equals the molecular weight of the fragment. For simplicity's sake, biologists often substitute base-pair length for molecular weight in this calculation.

- a. Orient your gel photo with the wells at the top, and working from bottom to top assign the known basepair sizes of the DNA standard to the bands appearing in the marker lane. Carefully measure the distance (in mm) each marker fragment migrated from the sample well. Measure from front edge of well to leading edge of each band.
 - b. Set up semilog graph paper with distance migrated as the x (arithmetic) axis and base-pair length as the y (logarithmic) axis. Then, plot distance migrated versus base-pair length for each marker fragment. Connect data points with a line.
 - c. Measure and record distances migrated by the PCR product. To determine its base-pair size, first locate the distance it migrated on the x axis. Then, use a ruler to draw a vertical line from this point to its intersection with the marker data line. Now, extend a horizontal line from this point to the y axis. The number on the y axis is the calculated basepair size of the amplification product.
 - d. How does the calculated size of the PCR product compare to its known size of 1100 base pairs?
3. Use the following information to calculate how many target DNA molecules were present in the 2.5 nanograms of *Lambda* DNA added to each PCR reaction:
 - One molecule of *Lambda* DNA is 48,502 basepairs (bp) in length.
 - One base pair has an average atomic mass of 650 grams/mole.
 - There are 6.02×10^{23} molecules per mole.

[a. Calculate the molecular weight of one molecule of *Lambda* DNA:

$$48,502 \text{ base pairs} \times 650 \text{ gram/mole per base pair} = 3.15 \times 10^7 \text{ gram/mole}$$

b. The 2.5 ng of *Lambda* DNA at the start of the experiment can be expressed as a fraction of a mole:

$$2.5 \times 10^{-9} \text{g} / 3.15 \times 10^7 \text{ g/mole} = 7.94 \times 10^{-17} \text{ moles}$$

c. Since there are 6.02×10^{23} molecules per mole, then the number of starting molecules is:

$$(7.94 \times 10^{-17} \text{ moles}) \times (6.02 \times 10^{23} \text{ molecules/mole}) = 4.78 \times 10^7 \text{ molecules}]$$

4. Calculate how many copies of the PCR product are produced following 17 cycles of amplification.

[a. If the PCR product doubles after each replication cycle, then 17 cycles will yield a theoretical amplification factor of $2^{17} = 1.31 \times 10^5$).

b. $1.31 \times 10^5 \times 4.78 \times 10^7 \text{ molecules} = 6.25 \times 10^{12} \text{ molecules.}]$

5. Judging from the results of your time course, what mass of DNA is the threshold of detection with ethidium bromide staining?

[a. Determine after how many cycles a PCR product is first faintly visible: usually 9 cycles.

b. Calculate the amplification factor at this number of cycles: 512.

c. Calculate the fraction of the starting mass of *Lambda* DNA (2.5 ng) represented by the 1100 bp target fragment:

$$\frac{1100 \text{ bp} (2.5 \times 10^{-9} \text{g})}{48,502 \text{ bp}} = 5.7 \times 10^{-11} \text{ g}$$

d. Multiply the amplification factor times the starting mass of the target fragment.

$$512 (5.7 \times 10^{-11} \text{ g}) = 29 \times 10^{-9} = 29 \text{ ng}$$

Ethidium bromide sensitivity, in fact, extends to approximately 2 ng of DNA.]



Amplifying Lambda DNA by Polymerase Chain Reaction (PCR)*

Student Guide

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- 1 to several minutes at 94-96°C, during which the DNA is denatured into single strands,
- 1 to several minutes at 50-65°C, during which the primers hybridize or "anneal" (by way of hydrogen bonds) to their complementary sequences on either side of the target sequence, and
- 1 to several minutes at 72°C, during which the polymerase binds and extends a complementary DNA strand from each primer.

As amplification proceeds, the DNA sequence between the primers doubles after each cycle. Following thirty such cycles, a theoretical amplification factor of one billion is attained.

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4. Calculate how many copies of the PCR product are produced following 17 cycles of amplification.
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