Biotechnology Explorer™

Cloning and Sequencing Explorer Series
Catalog #166-5000EDU

Planning Guide

explorer.bio-rad.com

Note: This document is for planning purposes only and may vary from the final product specifications.

For technical service, call our local Bio-Rad office or, in the U.S. call 1-800-424-6723

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Overview

Bio-Rad Laboratories will release the Cloning and Sequencing Explorer Series in Fall 2008. The intention of this planning guide is to provide an overview of the entire student research project including details of timelines, materials included, and equipment requirements so that instructors may prepare their curriculum and estimate budgetary requirements in advance. These are estimates based on current information available, and may change in the final version of the lab series.

The Cloning and Sequencing Explorer Series is comprised of eight lab modules which can be used separately or in series for an entire 6–8 week project. Due to the modular nature of the series, some components are used in conjunction with other modules for a continuous workflow.

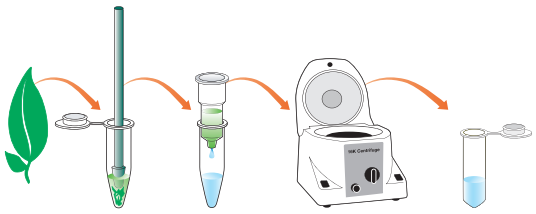
Cloning and Sequencing Explorer Series – All Eight Modules

166-5000EDU, supports 12 student workstations

Series includes:

166-5005EDU	Nucleic Acid Extraction Module
166-5010EDU	GAPDH PCR Module
166-0451EDU	Electrophoresis Module
732-6300EDU	PCR Kleen™ Spin Purification Module
166-5015EDU	Ligation and Transformation Module
166-5020EDU	Microbial Culturing Module
732-6400EDU	Aurum™ Plasmid Mini Purification Module
166-5025EDU	Sequencing and Bioinformatics Module
166-5001EDU	Curriculum CD, including Instructor's Guide, background information, student focus questions, graphic Quick Guides

Prepare DNA template using spin columns and centrifugation

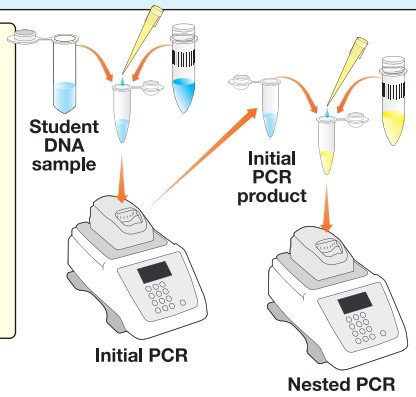


DNA extraction

Add master mix to student samples and positive control DNA

Amplify target sequences

Treat PCR sample with exonuclease I to remove primers



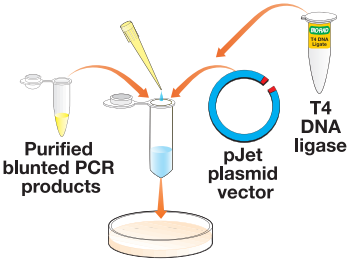
Add master mix and nested PCR primers to student initial PCR samples and positive control DNA

Amplify target sequences

Analyze via DNA electrophoresis

Two PCR steps and electrophoresis

Purify and blunt PCR products

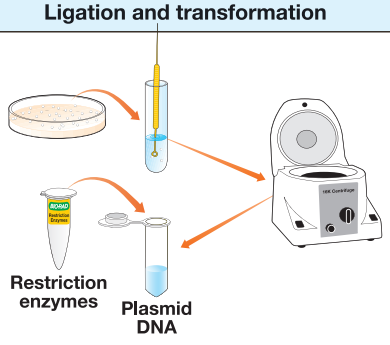


Combine PCR products, pJet plasmid vector, and T4 ligase. Ligate 10 minutes

Transform bacteria and incubate overnight at 37°C

Ligation and transformation

Grow liquid bacterial culture overnight at 37°C in shaking waterbath

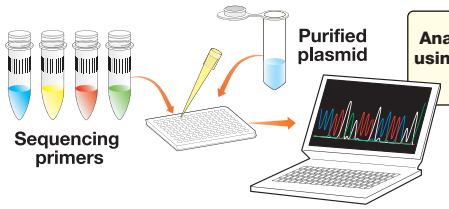


Purify plasmid DNA using alkaline lysis and miniprep spin column

Restriction digest analysis via DNA electrophoresis and positive clones

Miniprep: Restriction enzyme analysis and electrophoresis

Combine purified plasmid containing cloned gene with sequencing primers and mail to sequencing facility



Analyze sequences using bioinformatics software

Sequencing and bioinformatics

Abstract

Gene cloning techniques are a common topic in most molecular biology courses, but providing students with hands-on experience is more challenging. The objective of this project is to have students isolate, sequence, and characterize a pivotal gene of glycolysis (*GAPDH*, the gene for glyceraldehyde-3-phosphate dehydrogenase or *GAPDH*) from a plant species, or cultivar, with no previously published gene sequence. In addition to wet-lab techniques (e.g., DNA extraction, polymerase chain reaction (PCR), ligation, transformation, restriction digestion), students gain experience with bioinformatic analysis of their unique clones. This includes BLAST searches, identification of introns/exons, contig construction, and acquaintance with different bioinformatic databases. These results are publishable in the NCBI GenBank database.

Introduction and Course Fit

In this project students will isolate (clone) a major portion of the *GAPDH* gene. This gene is considered a housekeeping gene because it codes for an enzyme that catalyzes an important step of glycolysis, which is the stage of respiration that occurs in all living eukaryotic cells. Therefore, this gene is highly conserved and will be located in the genome of all plants.

GAPDH is a crucial enzyme for all animals, protists, fungi, bacteria and plants. Therefore, there are many opportunities to draw connections between the molecular aspects of *GAPDH* and its biomedical and evolutionary significance. Indeed, in Arabidopsis there are seven highly similar *GAPDH* genes. This lab targets a subfamily of these genes — *GAPC* and *GAPC2* — and students may clone one or both of these genes in the course of their experiment.

The basic strategy of this lab series is to extract genomic DNA and to synthesize large amounts of the DNA representing a portion of the *GAPDH* gene. This will be done using PCR on genomic DNA from a plant species you wish to study. Then you will blunt-end ligate this DNA to a cloning vector and transform *E. coli* cells with the resulting recombinant molecule. Surviving cells containing this recombinant DNA will be screened, assessed, and multiplied so that large quantities of recombinant DNA can be isolated and ultimately sequenced. The process of sequencing, typically done by a university or commercial lab, will reveal the exact sequence of DNA bases that makes up the *GAPDH* gene that was cloned. Once the exact DNA sequence is verified, this sequence can be published in the NCBI GenBank, the DNA database that researchers throughout the world consult.

This project is appropriate for the laboratory portion of an undergraduate (or early graduate) course in Molecular Biology, Cell Biology, Genetics, Biotechnology, Recombinant DNA Techniques, or Advanced Plant Biology. The project would also be suitable for students doing independent research. It takes a fairly sophisticated approach, so is geared more towards majors in Biology, Biochemistry, or Molecular Biology who are in their junior or senior year, or for graduate students in their first year. It would be excellent for inclusion in biotechnology degree programs offered by community or technical colleges. The exercise could also prove useful for employers in the biotechnology, pharmaceutical, or industrial sectors. This laboratory exercise is an effective way of demonstrating PCR, restriction enzymes, DNA vectors, ligation, transformation, recombinant bacterial screening, and bioinformatics for employees needing an introduction or a refresher course in biotechnology. Due to recent advances in the area of DNA technology, the actual laboratory procedures are routine, safe, and relatively inexpensive as long as basic cloning equipment is available. It is assumed that students meet at least once per week in a 3-hour lab session to cover all material in this 6–8 week exercise, and that there are other times during the week that students can meet to carry out a quick lab task or two.

The steps to be taken in this project are as follows:

1. Identify plant(s) to use and extract genomic DNA from plants.
2. Amplify region of *GAPDH* gene using PCR.
3. Assess the results of PCR.
4. Purify the PCR product.
5. Ligate PCR product into a plasmid vector.
6. Transform bacteria with the plasmid.
7. Isolate plasmid from the bacteria and analyze by restriction digestion.
8. Sequence DNA.
9. Perform bioinformatics analysis of the cloned gene.

Specific Objectives Met by this Project:

1. Students will experience a wide range of laboratory techniques. Some of the techniques implemented in this project are: DNA extraction and purification, PCR, nested PCR, gel electrophoresis, restriction enzyme digestion, working with cloning vectors, blunt-end ligation, preparation of competent cells, heat-shock transformation, subculturing, plasmid preparations, DNA sequencing, and bioinformatic analyses (e.g., BLAST searches).
2. Students will see that these individual techniques are steps in a longer investigatory process. Few researchers can complete an entire research project in one or two 3-hour lab sessions (the timeframe of most commercially available kits), so this 6–8 week project more accurately reflects the time scale in a contemporary molecular biology laboratory.
3. Students will be active participants in the process. There are numerous occasions during this project when students are asked to troubleshoot their results, or to make judgments about what to do next. This exercise does not take a simple 'cookbook' approach, but rather involves more critical thinking.
4. Students and their instructors will feel that the project is worthwhile. By having publication of a DNA sequence as the long-term goal of the project, both the students and the instructors will be more engaged in the process. This can make the experience more fulfilling (personally and professionally) than doing simulations.
5. This project is not an exercise where students are carrying out redundant experiments or are competing with each other in the lab. This exercise is a cooperative effort and requires students to share their data to create a larger, more useful, final product (a gene sequence from a single organism). On a larger scale, it is also possible for different institutions to cooperate. If different institutions set about to isolate the same gene from different species, and publish their sequences in the NCBI GenBank, then over the years an enormous database could be developed. Future students would have access to this large database to study the evolution of the gene using bioinformatic approaches.

Timeline for the Lab Course

The timeline will depend greatly on the level of the students, whether the ligation and transformation stages are combined or not, and whether other techniques and analyses are performed in addition to the basic protocol. A rough guide is provided here, and can be stretched or compacted depending on your schedule.

Lab Session	Chapter / Step	Task	Estimated Duration	Module Containing Materials
1	1: Nucleic acid extraction	Extract DNA	2 hr	Nucleic acid extraction
	2: *GAPDH* PCR	Set up initial PCR	0.5–1 hr	*GAPDH* PCR
	2: *GAPDH* PCR	Run PCR reaction in thermal cycler	3–4 hr*	*GAPDH* PCR
2	2: *GAPDH* PCR	Treat initial PCR reactions with exonuclease I	1 hr	*GAPDH* PCR
	2: *GAPDH* PCR	Set up nested PCR reactions	0.5–1 hr	*GAPDH* PCR
	2: *GAPDH* PCR	Run PCR reaction in thermal cycler	3–4 hr*	*GAPDH* PCR
	Prep for activities in chapter 3 Prep for activities in chapters 6 and 7	Pour agarose gels	0.5 hr	Electrophoresis Ligation and transformation, microbial culturing
		Pour LB and LB ampicillin IPTG agar plates	0.5 hr	
		Prepare LB and LB ampicillin broth	0.5 hr	
		Streak starter plate with bacteria	5 min	
		Grow starter plate at 37°C	16+ hr*	
3**	3: Electrophoresis	Electrophorese PCR products	0.5–1 hr	Electrophoresis
	3: Electrophoresis	Decide which PCR products to clone	0.5 hr	N/A
	4: PCR purification	Purify PCR products	0.5 hr	PCR purification
	Continue prep for activities in chapters 6 and 7	Complete prep from session 2	1 hr	Ligation and transformation, microbial culturing
		Inoculate single starter culture	5 min	
		Grow starter culture at 37°C	8+ hr*	

* Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or sequencing reactions to be processed.

** (Optional) Lab sessions 3, 4, and 5 can be combined into a single lab session.

Lab Session	Chapter / Step	Task	Estimated Duration	Module Containing Materials
4**	5: Ligation	Ligate PCR product	1 hr	Ligation and transformation
5**	6: Transformation	Transform bacteria with ligated product and plate them	1 hr	Ligation and transformation, microbial culturing
	6: Transformation	Grow bacteria at 37°C	16+ hr*	N/A
	Prep for activities in chapter 7	Pour agarose gels	0.5 hr	Electrophoresis
Between 5 and 6	7: Plasmid purification	Inoculate transformed colonies into miniprep LB ampicillin broth for culturing	5 min	Microbial culturing
	7: Plasmid purification	Grow bacteria at 37°C	8+ hr*	N/A
6	7: Plasmid purification	Purify plasmids from miniprep cultures	1 hr	Aurum™ plasmid mini purification
	7: Plasmid purification	Perform restriction digestion of plasmids	1–1.5 hr	Ligation and transformation
	7: Plasmid purification	Electrophorese plasmid digests	0.5–1 hr	Electrophoresis
7	7: Plasmid purification	Analyze results	1 hr	N/A
	8: Sequencing	Prepare sequencing reactions	0.5–1 hr	Sequencing and bioinformatics
	8: Sequencing	Send sequencing reactions away to be processed	Up to 2 weeks*	Sequencing and bioinformatics
8+	9: Bioinformatics	Practice analyzing sample sequences (optional)	3 h	Sequencing and bioinformatics
	9: Bioinformatics	Analyze sequences	6+ hr	Sequencing and bioinformatics

* Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or sequencing reactions to be processed.

** (Optional) Lab sessions 3, 4, and 5 can be combined into a single lab session.

Lab Session 1:

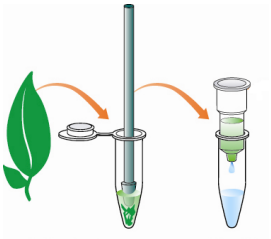
Lab Session	Chapter / Step	Task	Estimated Duration	Module Containing Materials
1	1: Nucleic acid extraction	Extract DNA	2 hr	Nucleic acid extraction
	2: *GAPDH* PCR	Set up initial PCR	0.5–1 hr	*GAPDH* PCR
	2: *GAPDH* PCR	Run PCR reaction in thermal cycler	3–4 hr*	*GAPDH* PCR

* Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or sequencing reactions to be processed.

Background

This project is an opportunity to perform novel research – to clone and sequence a gene that has not yet been analyzed and to add to the body of scientific knowledge. The first step in this exercise is to choose an interesting plant species to work with. Some model species that plant biologists study, for example *Arabidopsis thaliana, Chlamydomonas*, or crop plants like rice and wheat, have already had their genomes sequenced. You may choose to reproduce and confirm this sequence data. Alternatively, you may choose to select a species that is less studied. There are over 250,000 plant species known to exist on the planet, providing plenty of options. Also, you could choose a variety or cultivar (within a species) that no one has examined yet.

In order to clone a gene from an organism, DNA must first be isolated from that organism. This genomic DNA is isolated from one or two plants using column chromatography. Plant material is weighed, and then the material is ground in lysis buffer with high salt and protein inhibitors using a micropestle. The solid plant material is removed by centrifugation, then ethanol is added to the lysate and lysate is applied to the column. The ethanol and salt encourage DNA to bind to the silica in the chromatography column. The column is then washed and the DNA is eluted using sterile water at 70°C.



For PCR to be successful, the DNA extracted needs to be relatively intact. The best sources for DNA extraction are young green leaves, but fruit, roots, or germinating seeds should also suffice. It is better to use tissue that is still growing, as the nucleus:cytoplasm ratio will be more favorable, cells walls will be thinner, and the amount of potentially harmful secondary products will be less. There are two features of plants that make DNA extraction different from animals. First, plants have a tough cell wall made of cellulose that has to be penetrated. Second, a major part of every plant cell is a vacuole that contains acids, destructive enzymes (including nucleases), and unique secondary compounds (products produced from pathways that are not part of primary metabolism) that potentially damage DNA. To minimize contaminants from the vacuolar contents, salts and other inhibitors have been added to the lysis buffer.

Although every attempt has been made to make this lab as universal as possible to all plants, the fact that this lab uses a single DNA extraction method means there will be some plants for which the DNA extraction method does not succeed. These may be plants with very tough extracellular matrices or cell walls, or plants with some other characteristic that makes it difficult to extract DNA. Moreover, different plants will yield different quantities of DNA and the ability of that DNA to amplify may vary. For example, plants that yielded very little genomic DNA (gDNA) may amplify easily, while other plants that yielded a lot of gDNA may amplify

poorly. Likewise, PCR primers have been designed to amplify GAPDH from the majority of plants. However, although GAPDH is extremely conserved on the protein level, there is a good deal of variation among GAPDH DNA sequences of different plant species, so there may be some plants whose DNA amplifies poorly, or not at all, with the primers provided. Alternatively, a particular plant may contain a metabolite that interferes with PCR, preventing amplification.

Step 1–1: DNA Extraction

It is recommended that each student team extracts DNA from two plants and that the entire class uses the same two plants. To increase the chance of success, you could choose a plant known to work and a less well-characterized plant. If your goal is to obtain solid sequence data to be uploaded into GenBank, you are encouraged to have the entire class perform the research on the same plant. You will have multiple separately generated sequences, ensuring proper depth of coverage for the sequence.

- □ Select plant to be used (50–100 mg material required)
- □ Chop, lyse, grind plant material
- □ Purify on spin column

Nucleic Acid Extraction Module (catalog #166-5005EDU)

Kit contains sufficient materials for 12 student workstations, or 25 genomic DNA extractions.

Included in Module		Required Accessories	
Item	**Qty**	**Item**	**Qty**
Lysis buffer, 20 ml	1	Plant samples	varies
DTT (Dithiothreitol), 0.3 g	1	95-100% lab grade ethanol	1
Wash buffer, low stringency (5x), 20 ml	1	Adjustable micropipets, 20–200 µl	1/wkstation
Sterile water, 2.5 ml	1	Adjustable micropipets, 100–1000 µl	1/wkstation
Micropestles	25	Pipet tips, aerosol barriers, 20–200 µl	1 box/wkstation
Mini DNA extraction columns, purple	25	Pipet tips, aerosol barriers, 100–1000 µl	1 box/wkstation
Capless collection tubes, 2.0 ml	25	Water bath	1
Microcentrifuge tubes, 1.5 ml	30	Microcentrifuge capable of greater than 12,000 x g	2
Microcentrifuge tubes, multicolor, 2.0 ml	60	Balance and weigh paper	1
Instruction manual	1		

Optional: If time permits proceed directly to Step 1–2 and set up PCR reactions using freshly extracted genomic DNA.

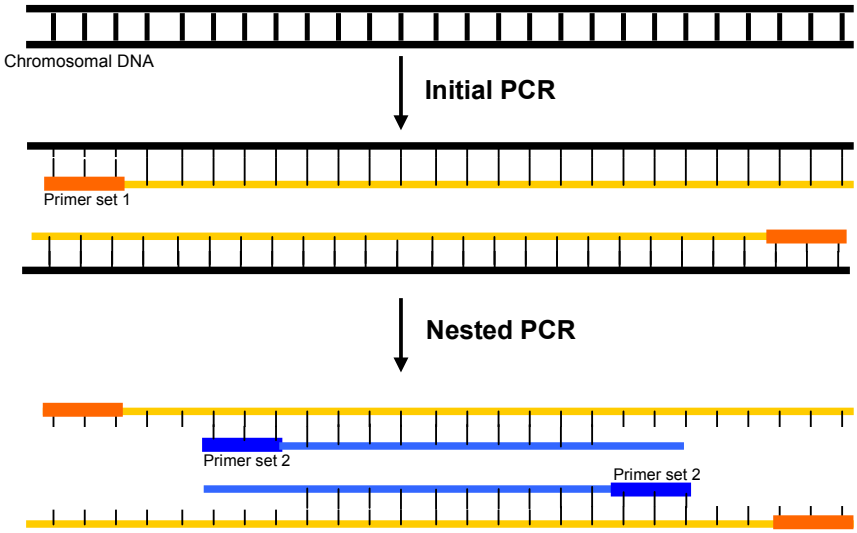
Optional: Dependent on time constraints, analyze samples prior to the next steps. This may include agarose gel electrophoresis, fluorometry, or spectrometry.

GAPDH PCR Background

The overall purpose of this experiment is to clone a portion of the glyceraldehyde-3 phosphate dehydrogenase gene (GAPDH). Because it is a vital metabolic enzyme involved in one of the most basic of biological processes — glycolysis in respiration — the GAPDH protein is highly conserved between organisms, especially vital domains of the enzyme, such as the active site. However, this does not mean that the gene and DNA sequence are identical in different organisms. Much of a gene does not code for protein; this “intronic” DNA is not subject to the same selective pressures as DNA that codes for protein. In addition, for the gene exons — gene sections that do encode for proteins — there is degeneracy of the genetic code such that different DNA triplet codons encode the same amino acid. Also some regions of

the enzyme less vital to function (other than an active site) do not have the same degree of selective pressure and although there is conservation of protein sequence, it may not be as stringent in some areas as others.

To clone a known gene from an uncharacterized organism, PCR primers (short synthetically synthesized, single-stranded oligonucleotides often 17–25 bases) must be designed that are complementary to conserved regions of *GAPDH* genes. However, even conserved regions are not identical between organisms. A best guess of the gene sequence is made using a comparison alignment from the sequences of *GAPDH* genes from different but related organisms, with the understanding that the primers will not be an exact match to the sequence and may amplify nonspecific sections of DNA in addition to the target sequence. A second set of primers is then designed (interior to the first set of primers) and used to amplify the PCR products from the first round of PCR. This technique is called "nested PCR" and is based on the extremely slim chance of nonspecifically amplified DNA also encoding these interior sequences, whereas the target sequence should contain these sequences, even if the match is not perfect. In other words, if the wrong fragment was amplified with the first primers, the probability is quite low that the wrong fragment will be amplified during the second round of PCR. As a result, the PCR products generated from nested PCR are very specific. Additionally, since the nested PCR primers are in the interior of the first fragment, the PCR products generated during the second round of PCR are shorter than the first one. See figure below for an illustration of nested PCR.



The products of the initial PCR reaction will be diluted and used as the templates for the nested PCR reaction. Arabidopsis genomic DNA has been included as a control for these PCR reactions. In addition, a plasmid encoding the targeted region of GAPDH from Arabidopsis will be used as a further control.

As each PCR reaction takes approximately 3–4 hours to run, it is most practical to run the PCR reactions on separate days. Since the reagents used in these experiments function optimally when prepared fresh, just prior to running the PCR reactions, it is highly recommended that the reagents be prepared just prior to setting up the PCR reactions.

Important note: PCR is extremely sensitive to contamination by DNA from many sources. All manipulations involving reagents to be used for PCR should be handled with care so that contamination is minimized. To avoid contamination, it is recommended that PCR reactions are set up in an area of the laboratory or classroom that is separate from the DNA extraction area, and/or that the lab benches are thoroughly swabbed down with a commercial

cleaner or 10% bleach (ethanol does not destroy DNA). In addition, aerosol barrier pipet tips should always be used to set up PCR reactions (and for preparation of template DNA). It is recommended that pipets be carefully cleaned with a 10% solution of bleach before performing PCR. In research labs, PCR flow hoods are frequently utilized to prevent contamination.

Step 1–2: GAPDH PCR

Plan the initial PCR experiment: one initial PCR reaction will be performed for each of the genomic DNA samples extracted.

- □ Prepare DNA samples for PCR
- □ Set up PCR reactions
- □ Run PCR

GAPDH PCR Module (catalog #166-5010EDU)

Kit contains sufficient materials for 12 student workstations.

Included in Module		Required Accessories	
Item	**Qty**	**Item**	**Qty**
Initial GAPDH PCR primers, 50 µl	1	Adjustable micropipets, 2–20 µl	1/wkstation
Nested GAPDH PCR primers, 50 µl	1	Adjustable micropipets, 20–200 µl	1/wkstation
PCR master mix, 1.2 ml	3	Pipet tips, aerosol barriers, 2–20 µl	1 box/wkstation
pGAP control plasmid DNA for PCR, 1 ml	1	Pipet tips, aerosol barriers, 20–200 µl	1 box/wkstation
5x Control Arabidopsis gDNA, 20 µl	1	Thermal cycler	1
Exonuclease I, 50 µl	1	Ice bath	1/wkstation
500 bp molecular weight ruler*, 400 µl	1		
Sterile water, 2.5 ml	1		
PCR tubes, 0.2 ml	150		
Capless PCR tube adaptors, 1.5 ml	150		
Microcentrifuge tubes, multicolor, 2.0 ml	120		
Foam microcentrifuge tube holders	12		
Instruction manual	1		

Optional: Although this protocol recommends analyzing the PCR products after both rounds of PCR have been completed, PCR results can be assessed using electrophoresis directly after this reaction is complete. Positive controls should yield visible bands. It is possible that some plant genomic DNA will not yield a visible band during the initial round of PCR and yet still be amplified after the second round of nested PCR. Note: If this is done — DO NOT add loading dye directly to the PCR reactions as loading dye may interfere with the subsequent round of PCR.

Lab Session 2:

Lab Session	Chapter / Step	Task	Estimated Duration	Module Containing Materials
2	2: *GAPDH* PCR	Treat initial PCR reactions with exonuclease I	1 hr	*GAPDH* PCR
	2: *GAPDH* PCR	Set up nested PCR reactions	0.5–1 hr	*GAPDH* PCR
	2: *GAPDH* PCR	Run PCR reaction in thermal cycler	3–4 hr*	*GAPDH* PCR
	Prep for activities in chapter 3 Prep for activities in chapters 6 and 7	Pour agarose gels	0.5 hr	Electrophoresis Ligation and transformation, microbial culturing
		Pour LB and LB ampicillin IPTG agar plates	0.5 hr	
		Prepare LB and LB ampicillin broth	0.5 hr	
		Streak starter plate with bacteria	5 min	
		Grow starter plate at 37°C	16+ hr*	

Background

In this next lab, PCR products generated in the previous step will be further amplified (i.e., serve as the template) in a second round of PCR. However, before performing the nested PCR, the primers that were not incorporated into PCR product must be removed so that they do not amplify target DNA in the second round of PCR. To do this, an enzyme that specifically digests single-stranded DNA, exonuclease I, will be added to the PCR reactions. After the initial PCR primers have been digested, exonuclease I also needs to be inactivated before it is introduced into fresh PCR reactions to prevent it digesting the nested PCR primers. In nature, this enzyme is involved with proofreading and editing newly synthesized DNA.

Following exonuclease I treatment, diluted PCR products from genomic templates generated in the first round of PCR will be amplified using the nested primers. Plasmid DNA will also be amplified in this step to serve as a positive control for PCR. A no-template negative control will also be run.

Step 2–2: Nested GAPDH PCR

- □ Treat PCR reactions from initial PCR with exonuclease I
- □ Heat-inactivate exonuclease I
- □ Prepare PCR reactions for 2nd round
- □ Run PCR

Prepare for electrophoresis, ligation, and transformation:
After the PCR reactions are completed they will be analyzed by agarose gel electrophoresis. In addition, bacterial culturing materials for steps 4, 5, and 6 need to be prepared.

- □ Prepare LB starter plates
- □ Streak out starter colonies of BH101 bacteria (at least two days prior to transformation)
- □ Prepare LB Amp IPTG agar plates
- □ Prepare sterile LB broth for step 5
- □ Cast agarose gels (materials from Electrophoresis Module, catalog #166-0451EDU)

Microbial Culturing Module (catalog #166-5020EDU)
Kit contains sufficient materials to pour 40 LB agar plates (with or without ampicillin), inoculate and grow 75 miniprep cultures; and starter *E. coli* bacteria.

Included in Module		Required Accessories	
Item	**Qty**	**Item**	**Qty**
Ampicillin, lyophilized	2	Microwave oven	1
LB broth capsules	12	Incubation oven	1
LB nutrient agar powder	1	Shaking water bath	1
IPTG*, 0.1 ml	1		
Petri dishes, 60 mm, sterile	40		
Cell culture tubes, 15 ml, sterile	75		
Inoculation loops, sterile	80		
E. coli strain HB101 K-12, lyophilized	1		
Disposable plastic transfer pipets	10		
Instruction manual	1		

* Time indicated for these tasks is not hands-on time. It is the time needed for reactions to run, bacteria to grow, or sequencing reactions to be processed.

Lab Session 3:

Lab Session	Chapter / Step	Task	Estimated Duration	Module Containing Materials
3	3: Electrophoresis	Electrophorese PCR products	0.5–1 hr	Electrophoresis
	3: Electrophoresis	Decide which PCR products to clone	0.5 hr	N/A
	4: PCR purification	Purify PCR products	0.5 hr	PCR purification
	Continue prep for activities in chapters 6 and 7	Complete prep from session 2	1 hr	Ligation and transformation, microbial culturing
		Inoculate single starter culture	5 min	
		Grow starter culture at 37°C	8+ hr*	

The fragment of *GAPDH* that has been targeted varies in size between plant species. The expected size of the fragment from the first round of initial PCR is expected to be 0.5–2.5 kbs. The expected size of the fragment from the second round of nested PCR is expected to be slightly smaller than the product from the initial round of PCR. It is likely that some plants may amplify multiple bands — which correspond to multiple *GAPDH* genes within the plant's genome. The nested PCR should result in a single band or doublet — corresponding to the *GAPC* and/or *GAPC2* gene of the organism. This is probably due to amplification of two *GAPDH* genes that are very homologous (genes that share similar structures and functions that were separated by a duplication event).

Step 3–3: Electrophorese PCR Products

□ Prepare PCR products for loading on gel
□ Load and run gels□ Prepare LB Amp IPTG agar plates
□ Analyze PCR data

Electrophoresis Module (catalog #166-0451EDU)

Included in Module		Required Accessories	
Item	**Qty**	**Item**	**Qty**
Agarose powder, 25 g	1	Horizontal gel electrophoresis chambers	4–12
Electrophoresis buffer, 50x TAE, 100 ml	2	Power supplies	3–6
UView loading dye and stain, 6x, 25 µl	1	Adjustable micropipets, 2–20 µl	1/wkstation
500 bp molecular weight ruler*, 400 µl	1	Pipet tips, aerosol barriers, 2–20 µl	1 box/wkstation
Microcentrifuge tubes for preparing samples and aliquoting	1	Microwave oven (for melting agarose to cast gels)	1

* From *GAPDH* PCR Module.

Choose a plant GAPDH to clone

Once the class has their results, it is time to pick a plant to clone. Although two plants were chosen to investigate, only a single plant's *GAPDH* gene will be cloned. It is recommended that the plant chosen be the one that generated the cleanest PCR product (fewest background bands), with good band intensity of an appropriate size. It is acceptable to clone doublets since each plasmid is expected to ligate a single DNA fragment. Be aware that two different gene sequences may be obtained from different minipreps.

It is highly recommended that the entire class clone *GAPDH* from the same plant, so that the data obtained will be more reliable. Cloning the same gene multiple times will provide significant coverage, which will help to resolve any ambiguous base pairs when the gene is sequenced. Remember, the ultimate goal of this laboratory is to provide new data for the scientific community at large, thus it is vital the data provided be as correct as possible.

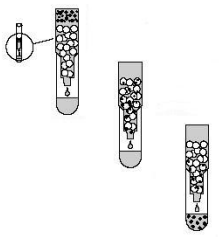
It is recommended that one or two groups perform an additional PCR purification, ligation, and transformation of the control Arabidopsis *GAPDH* PCR fragment, as a positive control for the class.

Step 3–4: Purification of PCR Products

□ Prepare columns for samples
□ Purify samples on spin column

Background

The next step after generating DNA fragments is to find a way to maintain and sequence these products. This is done by ligating (inserting) the fragments into a plasmid vector (small circular pieces of double-stranded DNA found naturally occurring in bacteria) that can be propagated in bacteria. To increase the success of ligation, it is necessary to remove unincorporated primers, nucleotides, and enzymes from the PCR reaction.



This is done by using size exclusion column chromatography. In size exclusion chromatography small molecules like proteins, primers, and nucleotides, get trapped inside the chromatography beads while large molecules, like DNA fragments, are too large to enter the beads and pass through the column into the microcentrifuge tube.

Without this cleaning step, we would be unsuccessful in the next steps of the cloning process: blunt-ending our PCR product with a proofreading polymerase, and ligating it into a vector. The opportunity also exists here to run gel electrophoresis of PCR product samples before and after cleaning to demonstrate the efficacy of the spin-column cleaning.

PCR Kleen™ Spin Purification Module (catalog #732-6300EDU)

Kit contains sufficient materials for 12 student workstations, or 25 PCR purifications.

Included in Module		Required Accessories	
Item	**Qty**	**Item**	**Qty**
PCR Kleen spin columns, clear	25	Adjustable micropipets, 20–200 µl	1/wkstation
Capless collection tubes, 2.0 ml	25	Pipet tips, 20–200 µl	1 box/wkstation
Microcentrifuge tubes, 1.5 ml	25	Microcentrifuge capable of greater than 12,000 x g	2
Instruction manual	1		

***Important note*:** PCR Kleen spin columns are designed to be used in variable-speed benchtop microcentrifuges capable of generating a force 735 x g.

Optional: Electrophorese 5 µl of the purified sample along with 5 µl of the unpurified sample on an agarose gel.

Additional tasks to perform prior to next stage

Starter cultures must be inoculated one day prior to the transformation with a starter colony from the HB101 LB agar starter plate. Incubate cultures with shaking overnight at 37°C.

Lab Session 4 and 5:

Lab Session	Chapter / Step	Task	Estimated Duration	Module Containing Materials
4**	5: Ligation	Ligate PCR product	1 hr	Ligation and transformation
5**	6: Transformation	Transform bacteria with ligated product and plate them	1 hr	Ligation and transformation, microbial culturing
	6: Transformation	Grow bacteria at 37°C	16+ hr*	N/A
	Prep for activities in chapter 7	Pour agarose gels	0.5 hr	Electrophoresis

** (Optional) Steps 4 and 5 can be combined into a single lab session.

Background

At this stage, the PCR product will be inserted (ligated) into a plasmid vector. The plasmid is supplied ready to use and has already been opened ready to receive the fragment. However, prior to ligating the fragment into the plasmid, the PCR fragment must first be treated to remove a single adenosine nucleotide that is left on the 3' ends of the PCR fragment by Taq DNA polymerase. This is performed by a proofreading DNA polymerase (enzymes with a 3' proofreading exonuclease domain that allows the polymerase to remove mistakes in the DNA strands). This polymerase functions at 70oC but not at lower temperatures, so it is not necessary to inactivate this enzyme after use.

Once blunted, the PCR fragment is combined with the plasmid. A T4 DNA ligase (an enzyme that catalyzes the formation of phosphodiester bonds between the 5'-phosphorylated PCR fragment and the 3'-hydroxylated blunt plasmid) is added and the ligation reaction is completed in 5–10 minutes.

During ligation, many different products are produced. In addition to the desired ligation product where the PCR fragment has inserted itself into the plasmid vector, the vector may religate, or the PCR product may ligate with itself. Relatively few of the DNA molecules formed during ligation are the desired combination of the insert and plasmid vector. To separate the desired plasmid from other ligation products and also to have a way to propagate the plasmid, bacteria are transformed with the ligation reaction. Bacteria naturally contain plasmids, and plasmid vectors are natural bacterial plasmids that have been genetically modified to make them useful for molecular biologists. In order to get a plasmid into bacteria, the bacteria must be made “competent”. The bacteria must be actively growing, ice-cold, and suspended in transformation buffer that makes them porous and more likely to allow entry of plasmids.

Bacteria are then actively grown in culture media and pelleted, cooled, and resuspended in transformation buffer two times to ensure they are competent. It is vital to keep bacteria on ice at all times. Bacteria are subsequently mixed with the ligation reaction and plated on warm LB ampicillin IPTG agar plates that will only permit bacteria expressing ampicillin resistance genes (encoded by the pJet1.2 plasmid) to grow. These plates also contain isopropyl ß-D-1- thiogalactopyranoside (IPTG), which induces expression of the ampicillin resistance gene. Plates are then incubated at 37°C overnight. To ensure the cells were made competent by this procedure, a control plasmid will also be transformed.

Steps 4–5 and 5–6: Ligation and Transformation

This stage requires preparation of the bacterial cells for transformation prior to performing the ligation reaction. This may be broken up over different lab sessions depending on whether or not lab sessions 4 and 5 are combined.

Note: It is very important to keep the bacteria on ice during this procedure.

□ Prepare bacterial cells and solutions for transformation
□ Perform blunting reaction on clean nested PCR product
□ Set up ligation reaction
□ Transform cells
□ Plate transformation onto LB IPTG Amp agar plates

Ligation and Transformation Module (catalog #166-5015EDU)

Kit contains sufficient reagents for 12 student workstations.

Included in Module		Required Accessories	
Item	**Qty**	**Item**	**Qty**
T4 DNA ligase, 10 µl	1	Adjustable micropipets, 0.5–10 µl	1/wkstation
2x Ligation reaction buffer, 100 µl	1	Adjustable micropipets, 20–200 µl	1/wkstation
Proofreading polymerase, 10 µl	1	Adjustable micropipets, 100–1000 µl	1/wkstation
pJet1.2 blunted vector, 10 µl	1	Pipet tips, 0.5–10 µl	1 box/wkstation
Bgl II enzyme, 55 µl	1	Pipet tips, 20–200 µl	1 box/wkstation
10x Bgl II reaction buffer, 1 ml	1	Pipet tips, 100–1000 µl	1 box/wkstation
C-Growth medium, 30 ml	1	Water bath	1
Transformation reagent A, 1.25 ml	4	Microcentrifuge	2
Transformation reagent B, 1.25 ml	4	Incubation oven	1
IPTG, 1M, 0.1 ml	1	Shaking water bath	1
Sterile water, 1 ml	1	Ice bath	1/wkstation
Microcentrifuge tubes, multicolor, 2.0 ml	120		
Microcentrifuge tubes, 1.5 ml	30		
Instruction manual	1		

Between Lab Session 5 and 6:

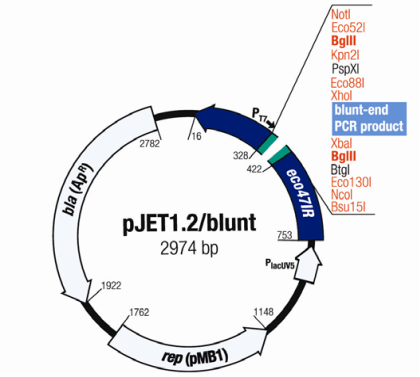
Lab Session	Chapter / Step	Task	Estimated Duration	Module Containing Materials
Between 5 and 6	7: Plasmid purification	Inoculate transformed colonies into miniprep LB ampicillin broth for culturing	5 min	Microbial culturing
	7: Plasmid purification	Grow bacteria at 37°C	8+ hr*	N/A

Lab Session 6:

Lab Session	Chapter / Step	Task	Estimated Duration	Module Containing Materials
6	7: Plasmid purification	Purify plasmids from miniprep cultures	1 hr	Aurum™ plasmid mini purification
	7: Plasmid purification	Perform restriction digestion of plasmids	1–1.5 hr	Ligation and transformation
	7: Plasmid purification	Electrophorese plasmid digests	0.5–1 hr	Electrophoresis

Background

It is necessary to analyze the plasmids that have been successfully transformed to verify that they have the PCR fragment inserted. To do so, a sufficient amount of plasmid DNA is obtained by growing a small culture of bacteria, purifying the plasmid from the bacteria, and performing restriction digestion (using an enzyme that cuts double-stranded DNA at specific recognition sequences) on the plasmids. This allows for assessment of the PCR fragment size that was inserted and comparison to the size of the PCR fragment ligated. The plasmid used to ligate the PCR products is pJet1.2 (see figure).



The blunted PCR product was inserted into the vector. pJet1.2 contains a BglII restriction enzyme recognition site on either side of the insertion site. Thus, once the plasmid DNA has been isolated, a restriction digestion reaction will be performed to determine the size of the insert.

Transformed bacteria containing plasmids should have been grown to saturation in LB ampicillin medium prior to this lab. In addition, an agarose gel is needed for the analysis of the restriction digest.

Step 6–7: Purify Plasmid Minipreps; Restriction Analysis of Plasmid DNA

- □ Centrifuge to pellet bacterial cells
- □ Resuspend bacteria; lyse open cells
- □ Spin-purify plasmids

Aurum Plasmid Mini Purification Module (catalog #732-6400EDU)
Kit contains sufficient reagents to purify DNA from 100 mini-cultures of plasmid-bearing bacteria.

Included in Module		Required Accessories	
Item	**Qty**	**Item**	**Qty**
Plasmid mini columns, green	100	Adjustable micropipets, 100–1000 µl	1/wkstation
Capless collection tubes	100	Pipet tips, 100–1000 µl	1 box/wkstation
Resuspension solution, 25 ml	1	95–100% lab grade ethanol	1
Lysis solution, 25 ml	1	Microcentrifuge capable of greater than 12,000 x g	2
Neutralization solution, 40 ml	1		
Wash solution, 5x, 25 ml	1		
Elution solution, 16 ml	1		
Instruction manual	1		

Step 6–7: Restriction Analysis of Plasmid DNA

- □ Set up restriction digest of plasmids
- □ Electrophorese samples

Student Workstation List		Required Accessories	
Item	**Qty**	**Item**	**Qty**
Purified plasmid DNA[1]	1	Horizontal gel electrophoresis chambers	4–12
Undigested plasmid DNA (optional)	1	Power supplies	3–6
Bgl II enzyme[2], 50 µl	1	Adjustable micropipets, 2–20 µl	1/wkstation
10x Bgl II reaction buffer[2], 1 ml	1	Pipet tips, 2–20 µl	1 box/wkstation
500 bp molecular weight ruler[3], 500 µl	1	Micro test tubes (for digestion and gel loading)	2/sample
UView loading dye and stain, 6x	1	Water bath	1
1% agarose gels[4]	1		
Refer to manual for preparation of gels and samples			

[1] From plasmid purification step.
[2] From Ligation and Transformation Module (catalog #166-5015EDU).
[3] From *GAPDH* PCR Module (catalog #166-5010EDU).
[4] Prepared from Electrophoresis Module (catalog #166-0451EDU).

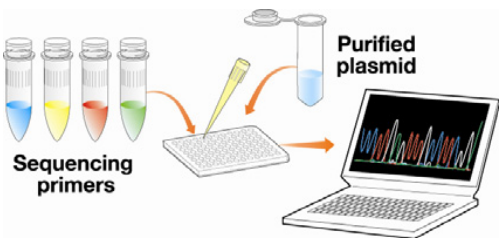
Optional: It is recommended that 5 µl of undigested DNA also be run next to your digested samples. Prepare these samples by combining 5 µl of miniprep DNA with 5 µl of sterile water.

Lab Session 7:

Lab Session	Chapter / Step	Task	Estimated Duration	Module Containing Materials
7	7: Plasmid purification	Analyze results	1 hr	N/A
	8: Sequencing	Prepare sequencing reactions	0.5–1 hr	Sequencing and bioinformatics
	8: Sequencing	Send sequencing reactions away to be processed	Up to 2 weeks*	Sequencing and bioinformatics

Background

The aim of this laboratory is to obtain sequence of a GAPDH gene from an unstudied organism. In addition, confirmation that the PCR product is GAPDH is required. To verify the PCR and to obtain novel sequence, the plasmids containing fragments suspected to be GAPDH genes need to be sequenced. Sequencing reactions rely on the basic principles of DNA replication like PCR and as such require primers to initiate the replication. However, sequencing is performed in just one direction and so instead of a primer pair, sequencing makes use of single oligonucleotides. Since a single sequencing run generates a read length of 500–700 base pairs (bp), multiple primers for sequencing in both directions are required to provide full coverage of the gene and to provide increased depth of coverage; thus, the same region is read in multiple reactions. The primers used for DNA sequencing are different from the primers used to amplify the GAPDH gene via PCR. Sequencing primers are designed to complement the DNA sequence of the cloning vector, rather than the insert DNA. If the primers used for PCR were also used for sequencing, then part of the clone's sequence would be missing, because sequencing starts about 20–50 bases away from the primer itself. This is a function of the size of DNA polymerase. Most commercially available cloning vectors are designed to have sites that are relatively far from the cloning region and that will bind to widely available sequencing primers. These universal sequencing primers allow researchers to work with different cloning vectors at the same time. However, the current method used in DNA sequencing only generates ~500 bp of usable DNA bases, whereas the GAPDH gene in some species is much larger. Therefore, internal sequencing primers are used to primer-walk (the primers are designed to obtain a contiguous sequence from an internal region of the gene of interest). In our case, doublestranded primer walking (providing contiguous sequence information from both DNA strands) will be performed. In this lab, four sequencing reactions for each plasmid will be run — two in the forward direction and two in reverse. Two primers are to the vector sequence on either side of the PCR product and two primers have been designed to anneal to conserved regions within the GAPDH PCR product. The plasmid DNA will be combined with the sequencing primers and then mailed to a sequencing laboratory, which will perform the sequencing reactions, analyze the results, and send the DNA sequence back for analysis.



The technique for determining the exact order of As, Ts, Cs, and Gs in cloned DNA is called the dideoxy or Sanger method, named for Dr. Fred Sanger of Cambridge, England, who invented it in the mid-1970s. In this approach, the plasmid clones are heat-denatured (to separate the complementary DNA strands using high temperatures) and used as template to synthesize new strands of DNA. To do this, the template is incubated with DNA polymerase, sequencing primers, deoxynucleotide triphosphates (dNTPs: dATP, dTTP, dCTP, dGTP) and relatively small amounts of

dideoxynucleotide triphosphates (ddNTPs: ddATP, ddTTP, ddCTP, ddGTP). The difference between the deoxy- form and the dideoxy- form of a nucleotide triphosphate is a missing -OH group on the 3' carbon of the deoxyribose. This missing -OH (hydroxyl) group is necessary for normal DNA synthesis, so if a growing chain of DNA happens to utilize a ddNTP, instead of a dNTP, the DNA synthesis reaction is stopped. This termination event occurs rarely enough that all possible lengths of DNA get synthesized during the process. For instance, a sequencing reaction that provides the DNA sequence for 700 bases would essentially have involved synthesizing 700 different strands of DNA, covering the entire range of possible lengths. These different lengths of DNA are resolved by electrophoresis and visualized. A common visualization method is to 'end-label' each of the four types of ddNTPs with a different label, originally a radioisotope that can be distinguished after electrophoresis. The Sanger method is now routinely modified to use a fluorescent dye to end-label the ddNTP, followed by dye detection with a digital camera after capillary electrophoresis. This approach detects and records the dye fluorescence and shows the output as fluorescent peaks on a chromatograph. DNA sequence output is based on the fact that longer strands of DNA move more slowly during electrophoresis than shorter lengths, and that the digital camera can detect the color of the fluorescent dye that labels each of the bands. Since the specific color of the dye attached to each of the different ddNTPs is known, and since that specific ddNTP will end-label the growing DNA strand on the plasmid template, the task of correlating the order of colors with a specific sequence of DNA is relatively straightforward.

Step 7–8: Set up Sequencing Reactions

- □ Assign student teams to groups of wells on the class 96-well plate
- □ Record barcode of 96-well plate for tracking purposes
- □ Label tubes to correlate with wells on plate
- □ Combine purified plasmid DNA with sequencing primers
- □ Add samples to plate; seal plate
- □ Send plate to sequencing facility

DNA sequences will be obtained either through the Joint Genome Institute (JGI) or from a local DNA sequencing service.

Sequencing and Bioinformatics Module (catalog #166-5025EDU)
Kit contains reagents sufficient materials for 12 student workstations, or 96 sequencing reactions, and a three month user license for Geneious genetic analysis tools.

Included in Module		Required Accessories	
Item	**Qty**	**Item**	**Qty**
pJET SEQ F primer, 50 µl	1	Adjustable micropipets, 0.5–10 µl	1/wkstation
pJET SEQ R primer, 50 µl	1	Pipet tips, 0.5–10 µl	1 box/wkstation
GAP SEQ F primer, 50 µl	1	Computers with Internet access	
GAP SEQ R primer, 50 µl	1		
pGAP control plasmid for sequencing, 100 µl	1		
Barcoded 96-well plate	1		
Sealing film	1		
Microcentrifuge tubes, multicolor, 2 ml	120		
Instruction manual	1		

Lab Session 8:

Lab Session	Chapter / Step	Task	Estimated Duration	Module Containing Materials
8+	9: Bioinformatics	Practice analyzing sample sequences (optional)	3 h	Sequencing and bioinformatics
	9: Bioinformatics	Analyze sequences	6+ hr	Sequencing and bioinformatics

In this part of the laboratory, DNA sequences will be run through a series of analyses. This portion of the laboratory is quite open-ended: the level of complexity and the depth of the analyses are entirely up to the instructor. Time constraints may prevent following all steps in the process, but the following types of analyses are suggested:

1. Identify the cloned *GAPDH* sequences.
2. Use the sequences to perform a BLAST search on the NCBI GenBank nucleotide database.
3. Assemble sequences into a contig using the CAP3 program.
4. Identify introns and exons (including addition of annotations).
5. Predict mRNA sequence and check with BLAST.
6. Translate the mRNA sequence to predict the sequence of the proteins.

Overview

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Cloning and Sequencing Explorer Series: Modules and Equipment Requirements

EQUIPMENT AND ACCESSORY REQUIREMENTS	1665000EDU: Cloning & Sequencing Explorer Series All 8 Modules	1665005EDU: Nucleic Acid Extraction Module	166-5010EDU: GAPDH PCR Module	166-0451EDU: Electrophoresis Module	732-6300EDU: PCR Kleen Spin Purification Module	166-5015EDU: Ligation and Transformation Module	166-5020EDU: Microbial Culturing Module	732-6400EDU: Aurum Plasmid Mini Purification	166-5025EDU: Sequencing and Bioinformatics Module
Required accessories not included in kit									
Micropipets: 0.5-10 µl (166-0505EDU, 166-0550EDU)	1 - 12					1 - 12			
Micropipets: 2-20 µl (166-0506EDU, 166-0551EDU)	1 - 12		1 - 12	1 - 12					1 - 12
Micropipets: 20-200 µl (166-0507EDU, 166-0552EDU)	1 - 12	1 - 12	1 - 12		1 - 12	1 - 12			
Micropipets: 100-1000 µl (166-0508EDU, 166-0553EDU)	1 - 12	1 - 12				1 - 12		1 - 12	
Pipet tips, aerosol barrier, 2-20µl (211-2006EDU)	1 box/wkst		1 box/wkst						
Pipet tips, aerosol barrier, 20-200 µl (211-2016EDU)	1 box/wkst	1 box/wkst	1 box/wkst						
Pipet tips, aerosol barrier, 100-1000 µl (211-2021EDU)	1 box/wkst	1 box/wkst							
Pipet tips, standard style, 0.5-10 µl (223-9354EDU)	1 box/wkst					1 box/wkst			
Pipet tips, standard style, 2-200 µl (223-9347EDU)	1 box/wkst			1 box/wkst	1 box/wkst	1 box/wkst			1 box/wkst
Pipet tips, standard style, 100-1000 µl (223-9350EDU)	1 box/wkst					1 box/wkst		1 box/wkst	
Microcentrifuge (>12k x g, 166-0602EDU)	1	1			1	1		1	
Water bath (166-0504EDU)	1	1	1			1			
Thermal cycler (170-9701EDU)	1		1						
Mini-Sub Cell GT (166-4000EDU)	4 - 12			4 - 12					
PowerPac Power Supply (164-5050EDU)	3 - 6			3 - 6					
Incubation Oven (166-0501EDU)	1					1	1		
Shaking water bath (166-0560EDU)	1					1	1	1	
95-100% lab grade ethanol	x	x						x	
Balance (capable of weighing 50 mg)	1	1							
Microwave oven	1			1			1		
Computers and Internet access	1 - 12								1 - 12
Recommended (optional) accessories									
Microcentrifuge(s)*			x						
Mini-Sub Cell GT (166-4000EDU)*					x				
PowerPac Power Supply (164-5050EDU)*					x				
Vortexer (166-0601EDU)	x	x	x						
Spectrophotometer (170-2525EDU)	x	x						x	
VersaFluor fluorometer (170-2402EDU)	x	x						x	
DEPC-treated water, 100 ml (700-7253EDU)	x	x							
Gel documentation system	x		x	x	x				
Aurum vacuum manifold (732-6470EDU)	x							x	

General lab equipment and materials needed:

Razor blades/scalpels, permanent markers, ice buckets, micro tube racks, graduated cylinders, erlenmeyer flasks

*These optional items are required for other modules.

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