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PCR and Cloning: A Lab Manual

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by

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HONORS THESIS ABSTRACT

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ABSTRACT (100-200 WORDS):

This lab manual will give students an introduction to some basic laboratory procedures used in PCR and cloning. Standard lab protocols often assume the technician already knows certain basic lab techniques. This manual will prepare students to follow these protocols and start designing experiments on their own. The theory behind PCR and cloning is stressed in this manual, because a clear understanding of the processes involved is essential for experimentation. It allows the student to pin-point any flaws in a procedure, leave out any unnecessary steps, and begin to develop their own methods. These are all signs of a thinking technician. Practice protocols with explanations of terminology and techniques are included in the manual as well as a trouble shooting guide.

I would like to thank

Dr. Johns, my professor

Dr. Scott Grayburn, the sequencing guy

Jenny, my lab assistant

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and Dr. Mitch Altschuler

Without their help and guidance, this manual would never have been possible.

PCR and Cloning: a lab manual

by Katherine Mason

Table of Contents

Introduction:

My goal in writing this book is to give beginning students an introduction to some basic laboratory procedures used in PCR and cloning. Standard lab protocols often assume the technician already knows certain basic lab techniques. This manual will prepare students to follow these protocols and start designing experiments on their own. The theory behind PCR and cloning is stressed in this manual, because a clear understanding of the process is essential for experimentation. It allows the student to pin-point any flaws in a procedure, leave out any unnecessary steps, and begin to develop their own methods. These are all signs of a thinking technician. Practices protocols with explanations of terminology and techniques are included for students as well as a trouble shooting guide.

General Theory:

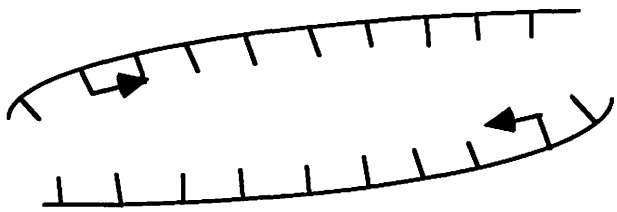
In Dr. Johns' lab, we have been trying to improve several RAPD primers from Cargill Seeds. Using these primers, we can distinguish between two lines of corn. Developing such primers allows for quick genetic analysis of any strain of corn, rather than having to perform crosses, backcrosses, and statistical analysis on many offspring to determine the genotype. RAPD (Random Amplified Polymorphic DNA) primers are 10-12 base pairs long, smaller than standard primers. They are so named because they bind to random sites in the corn genome. These primers, with the aid of Taq polymerase, can amplify segments of corn DNA in a reaction known as a PCR. When the resulting DNA is run on an electrophoresis gel, the amplified segments appear as a well-defined band. The presence of a band can indicate the strain of the corn.

Our job is to extend these short primers to make them less "random". Our approach is two-fold. Using PCR we can add bases onto the primers to make the bands brighter, better defined, or obtainable at higher annealing temperatures that would exclude other background bands. Whichever additional nucleotides improve the PCR outcome are valid additions to the primer. The other approach to extending the primers involves cloning. We take the amplified products of a PCR reaction and insert them into a plasmid vector. Then we transform the vector into bacteria and let them grow. Finally we isolate the cloned plasmids from the bacteria. Nested within the plasmids, the inserts are accessible for sequencing. Sequencing provides us with the exact nucleotide order which allows us to design longer, more specific primers.

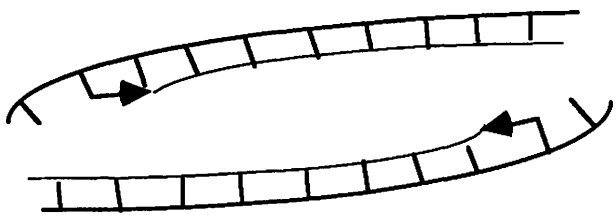
Theory of PCR:

The first half of this lab manual will deal with how to carry out PCR reactions. PCR stands for Polymerase Chain Reaction. It is used to refer to the amplification of segments of template DNA by primers in a thermal cycling reaction. Template DNA, in our case, is genomic DNA from different lines of corn. The reaction has three steps: heat cleaving of the double stranded DNA template, binding of the primers to the single stranded template strands, and addition of nucleotides to the 3' end of the primers by Taq polymerase. Taq is a heat tolerant enzyme isolated from Thermus aquaticus which is a bacterium found in hot springs. The three steps are repeated between about 35 times, replicating the segments of DNA between primers exponentially. See illustration.

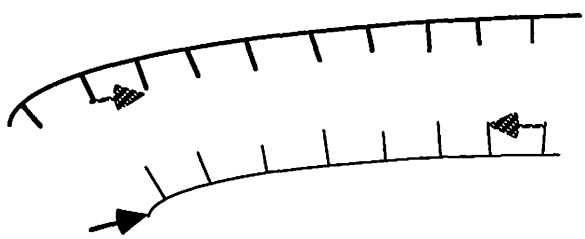
When we find a RAPD primer that provides a useful marker to distinguish between lines of corn, the first step is to extend that primer. We can make primers in the synthesis and sequencing lab (room 303). Just fill out the DNA Synthesis Request form for four new primers: the original primer plus A, T, C, and G. Then repeat the PCR reaction and see if one of the new primers works better than the original (gives a brighter, sharper band). The primer can be extended base by base making it less and less random.



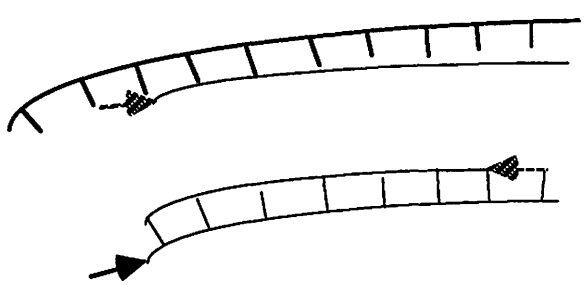
First the primers (arrows) bind to the template DNA



Then Taq polymerase adds base pairs to the 3' end of each primer



In the second cycle, the new strand separates from the original template and additional primers bind (only top strand is shown)



This time, when Taq adds new base pairs, a small segment of DNA is amplified (lower illustration)

How to Pipet:

We have four types of pipetmen in the lab. They can be distinguished by the labels on the top of their pistons. The color of the labels (white, yellow, and blue) correspond to the color pipet tip to use. The white tipped gun is used to pipet volumes under 10µl. The yellow ones are either labeled 20 or 200. The 20 is used to pipet volumes under 20µl. The 200 is used for volumes under 200µl. The blue one is used for volumes up to 1000µl (1 ml).

Turn the dial on the pipetman to select the volume you want to pipet
Wedge a pipet tip of the appropriate color onto the pipetman
Depress the piston until you feel resistance and insert the tip into your solution
Slowly release the piston to draw the liquid into the pipet tip
(make sure there are no air bubbles)
Depress the piston again to eject the liquid
pushing down hard will eject the last few drops of liquid

Preparing Solutions:

To prepare solutions from powder use the following formula:

Formula Weight (g/mol) x desired molarity (mol/L) /1000 = grams per ml

Sample calculation: making 100ml of 1M Tris
on the bottle: FW = 121g/mol
121g/mol x 1mol/L /1000 = 0.121g/ml
0.121g/ml x 100ml = 12.1g Tris powder in 100ml H_2O
Note: This means a TOTAL VOLUME of 100ml

To dilute existing solutions use the following formula:

$$C_1V_1 = C_2V_2$$

C_1 is the concentration you HAVE and C_2 is the concentration you WANT. V_2 is the volume you want. Solving for V_1 gives you the amount of your existing solution to add. You then add enough H_2O to bring the volume up to V_2.

Sample calculation: diluting 1M Tris to make 0.1M Tris
$C_1V_1 = C_2V_2$
$1M \times V_1 = 0.1M \times 100ml$
$V_1 = 10ml$ (of 1M Tris in 100ml of H_2O)

To make % solutions, such as 50% sucrose:
This is done by weight: 50 grams of sucrose and 50 grams of H_2O.
Note: 1gram H2O = 1ml H_2O

Setting up PCR Reactions:
PCR reactions are set up in small epindorf tubes. A maximum of 48 tubes can fit in the thermal cycler at one time. Each tube must contain four ingredients: Template DNA, Primers, Taq, and buffer with nucleotides in the following volumes:

5µl Template
5µl Primer
10µl Taq
5µl Buffer

If you want to use two primers, use 5µl of each and half as much Taq (just make it twice as concentrated). Most of the time it is more convenient and accurate to mix the Template, Taq, and buffer just before use. Then combine 20µl of this master mix with 5µl primer in each reaction tube.
After preparing the PCR mixture, add 50µl oil to each tube. The oil helps to distribute heat in the thermal cycler. Spin the tubes down for a few seconds in the microcentrifuge, making sure the tubes are balanced against each other, and you're ready to take them to the thermal cycler.

Template DNA is usually provided by Cargill. 25ng per reaction tube is sufficient for PCR reactions. Dilute the stock DNA to 5ng/µl (make 1ml at a time). "Home grown" template must also be diluted to 5ng/µl. It is made by growing corn from seeds in the greenhouse and extracting the DNA. Many plants can be done quickly with a kit, or a more lengthy CTAB extraction can be performed for larger volume isolations. See appendix.

Primers come in tubes from the sequencing lab or from Cargill. They must be diluted to 1µM before use.

Primer Dilution Protocol:

Determine the extinction coefficient (e) of the primer. This is done by multiplying the e for each base by the number of times that base appears in the primer. The e for the nucleotides are as follows:

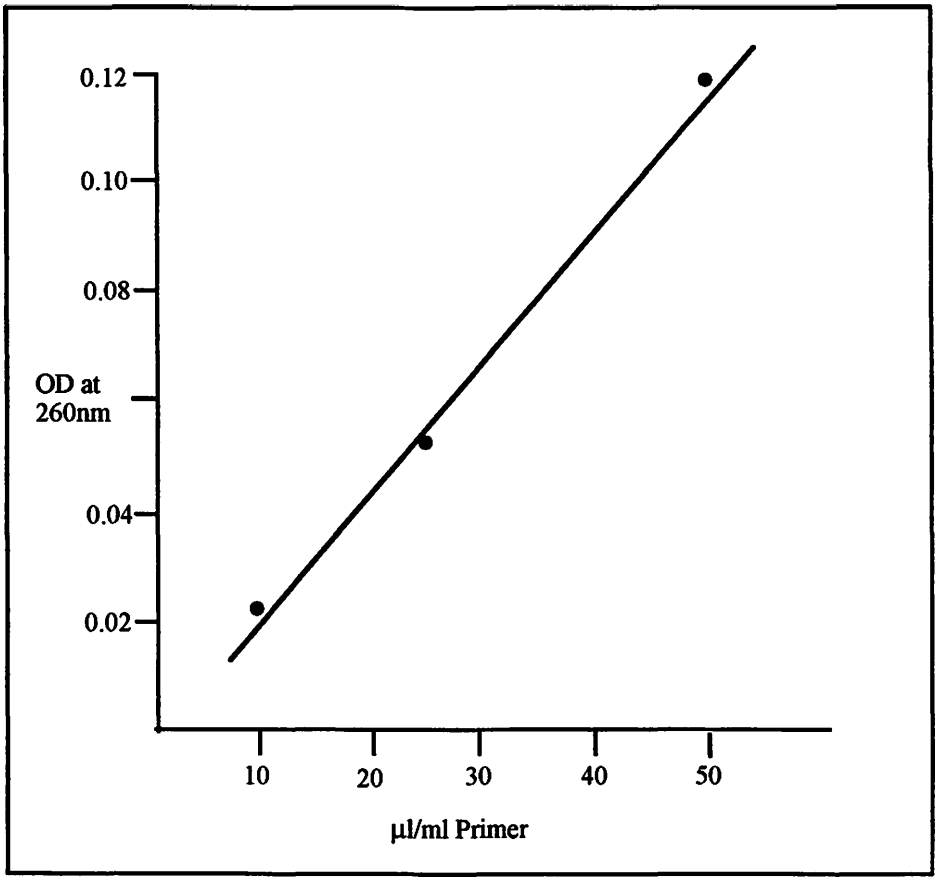
A = 15.4 L/mmol
C = 7.3
G = 11.7
T = 8.8

Sample calculation:
primer 556 is ATGGATGACG
A = 15.4 x 3 = 46.2
C = 7.3 x 1 = 7.3
G = 11.7 x 4 = 46.8
T = 8.8 x 2 = 17.6
e of primer 556 = 117.9

Add 1ml of sterile H20 to the dried primer. Get the H20 from room 303 and autoclave it. (See Autoclaving on page 11)
To measure the absorbance (OD) at 260nm, do the following steps:
Turn on the spectrophotometer and UV lamp and let it warm up
Make three dilutions of the primer: 10µl/ml, 25µl/ml, and 50µl/ml
Tare the spectrometer with the same water used for the dilutions
Take OD readings at 260nm on both sides of the cuvette.
(If the two readings are different, wipe off the glass surface)

ODs for primer 556:
10µl/ml = .023
25µl/ml = .054
50µl/ml = .120

Graph the OD on the Y-axis and the µl/ml primer on the X-axis



Find the slope of the graph and multiply by 1000 to get the OD of the full strength primer solution. The above graph for 556 primer gives a slope of .0024 OD/µl. Multiplying by 1000 gives us 2.4 OD.

Find the concentration of the primer using the following formula:

$$C = OD/e$$

where e is the primer's extinction coefficient you calculated previously.

For primer 556 the concentration is calculated to be 0.02mM

Determine the amount of full strength primer needed to make a 1µM solution by taking the reciprocal of the concentration.

For 556 primer, we will use 50µl/ml.

Taq is kept in the freezer. Dilute Taq fresh for every reaction: 40µl in 1ml H_2O.

Buffers provide ideal pH and salt concentrations for Taq. The buffer recipe we use is as follows:

50mM Tris pH 8.5
250mM KCl
7.5 mM $MgCl_2$
250µM dNTPs

To make 2ml:
100µl 1M Tris
38µl 1M HCl
500µl KCl
15µl 1M MgCl
20µl dNTPs (ATCG nucleotide mix)
400µl 50% sucrose
400µl 0.2% cresol red
in 2ml H_2O

The dNTPs are kept in the freezer in four separate tubes. Each tube is at a 100mM concentration. Mix equal volumes of the four to make a 25mM nucleotide solution of about 1ml. Avoid frequent freezing and thawing of the nucleotides. Sucrose and cresol red are used for convenience in loading the PCR product on a gel. The sucrose weighs down the DNA so it does not float away in the electrophoresis buffer and the cresol red is a dye. It is recommended to use more than one buffer for each PCR reaction. The pH can be varied between 8.5 and 8.6 and the magnesium concentration between 5 and 10mM.
Once you combine the four components in as many as 48 small tubes and add oil, you are ready to take your tubes to the thermal cycler.

The Thermal Cycler:

We use the Perkin Elmer Cetus DNA Thermal Cycler for our PCR reactions. There is one in room 412, one in 410, two in 317, and one in 323. The machines will hold 48 tubes at once. Once in the machine, the tubes are put through three linked files:

Time delay file: 94º for 10 min.,

35 cycle step-cycle file: 94º for 1min, 37º for 1min, and 72º for 1min.

Soak file: 4º indefinitely (i.e. until you show up to get them).

Since the files are linked, you only need to enter the Time delay file # and the thermal cycler will do the rest.

Thermal Cycler Operation
Turn machine on and insert tubes
Four keys: FILE, ##, ENTER, and START
Machine will begin counting down from 10:00 and heating to 94º

Time delay file ##s

Room	File #	Annealing Temperature
412	17	37º
410	27	35º
	21	37º
	23	39º
	30	45º
317	91	37º
323	40	37º
	42	39º

Programing the Thermal Cycler:

If you need to change your annealing temperature, you can just temporarily change your regular file. You can do this by typing in the step-cycle file number, pressing enter, and then pressing step to go through the program. When you get to the temperature, type in the new temperature and push enter. After you step through the whole program you can save it. Dr. John's user number is 1752. Just remember to change the program back to your original later. If you do not know the step-cycle file number, you can step through the time delay program and see to which file it is linked.

Pouring the Gel:

Gels are made with TBE buffer (Tris, Boric acid, and EDTA), 1.5% agarose, and 0.005% ethidium bromide. EtBr is a cancer causing chemical so it is recommended that you wear gloves. 5x TBE can be made four liters at a time and diluted as needed (400ml 5x TBE in 2L H_2O).

5x TBE buffer
216g Tris
110g boric acid
15g EDTA (or 80 ml 0.5M EDTA)
in 4L H_2O.

Large Gels

3.75g agarose
in 250 ml 1x TBE buffer
add 12.5 µl ethidium bromide (EtBr)

Small Gels

1.5g agarose
in 100ml 1x TBE buffer
add 5 µl ethidium bromide

Microwave in a glass flask until agarose is dissolved (2 or 3 minutes)
While you are waiting, tape the edges of the gel box
Run flask under cold water until it is cool enough to handle
Add ethidium bromide
Pour into taped gel casting tray
Flame out bubbles with Bunsen burner or comb them away
Insert the appropriate combs and wait about 15 minutes for the gel to harden

Loading the Gel:

Remove the combs and tape and place the gel box in the electrophoresis box
Cover the gel with 1x TBE buffer (See above recipe)
Insert your pipet tip below the oil and withdraw only the reaction mixture.
Carefully pipet the PCR samples into the submerged wells.
Load 5µl of 100bp ladder in left-most well of each row
(Add 50µl of enclosed dye to the full tube of ladder for better visibility)
Plug in the wires and turn on the electrophoresis machine (100- 150V).
Run large gels for about an hour, small gels for about 20 minutes.

Taking Pictures of Gels:

We use Polaroid 30 second processor to keep a photographic record of our gels.
To take pictures:
Place your gel on the UV light box
Place the appropriate sized light shield over your gel
Place the camera in the groove at the top of the light shield
Hold the camera steady and squeeze the trigger
Pull the film out of the camera
Wait 30 seconds and then peel the backing off the picture

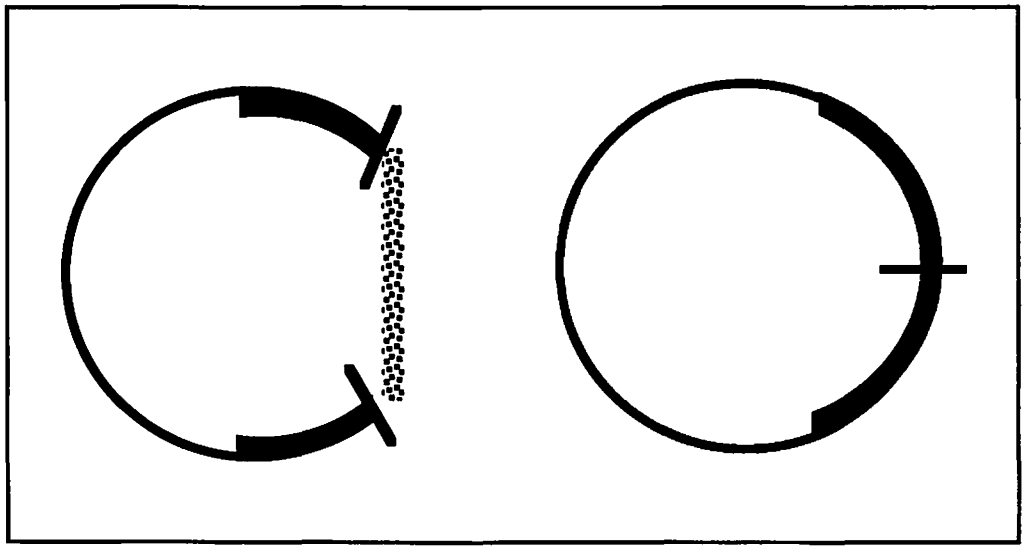
Camera Settings:

You can leave the speed at 1/2s (bottom dial) and change the F-stop depending on the size of your gel.

F-stop settings
Large gels = 4.5,
Small gels = 8 - 11

Theory of Cloning:

The second half of the lab manual is devoted to cloning. Cloning is a way to get many copies of a desired sequence of DNA. We do this by excising the desired band from a PCR gel, inserting it into a pGEM Easy Vector, and growing this plasmid in E.coli bacteria. Once the bacteria have grown into a culture of billions, we can isolate the plasmids and cut our DNA out with an enzyme.



This illustration shows a plasmid with an insert (left) and a plasmid without an insert (right). The thick part of the plasmid represents the ß-Galactosidase gene. When this gene is expressed in the presence of X-gal, a bright blue dye is produced by the bacteria. When the gene is interrupted by an insert, no dye is produced. If we only take white colonies, we can select for colonies with inserts. This is called blue/white screening.

One thing to be aware of during cloning procedures is to sterilize everything. This means autoclaving all solutions, tubes, flasks, pipet tips, and wiping down your work area with 70% ethanol. This prevents foreign bacteria from getting into your plates and nucleases from getting into your DNA thus ruining your results.

Operating Instructions for 4th floor Autoclave
Turn on red "light switch" and let the autoclave warm up for 15- 20 minutes
Put your stuff in (loosen caps, put foil over tops of flasks)
Close door
Close gray escape valve on top
Open black steam valve below
Wait about 1/2 hour (must hold at 15 psi for 20 min.)
Close black steam valve
Open gray escape valve
Wait until pressure lowers to 1 psi
Open door (avoid steam)
Take out stuff with hot mitts
Turn off "light switch" when you are done

The 3rd floor autoclave is more automated. There are no valves. Just put your stuff in and set the left and right displays to 20 min and 5 min respectively. 5 min is the cool down time. Cooling down faster may cause bottles to break.

Preparing DNA for use with pGEM-T Easy Vector::

The first step in cloning is to produce the desired band by PCR and then purify the band DNA by ethanol precipitation.

Perform a PCR reaction of four to eight tubes as described earlier. Run the DNA on a small gel until you can see your band glowing brightly. Cut the end off of one or more filter tips so they will fit inside large epindorf tubes. Get a sterilized scalpel or razor blade and wipe it with 70% ethanol. Reach under the UV light shield and cut the band out of the gel, trimming away all excess gel. Carefully put the slices of band into the filter tips. Spin the tips down in the ultracentrifuge for 10 minutes. The filter will let your DNA through, but will retain the agarose. Remove the excess liquid in the filter tip with a pipetman. This DNA is now ready for ethanol precipitation.

Ethanol Precipitation
Find the volume of your DNA
Add: 0.1 x vol. 5M KoAc
2.2 x vol. 100% ethanol
1µl glycogen
Freeze for at least 15 min at -80° (room 410)
Spin in ultracentrifuge at least 10 min (room 410)
Drain off supernatant
Add 500µl 70% ethanol
Vortex briefly and spin for another 10 min
Drain away the ethanol
Dry the pellet in speed vac if possible
(See appendix for speed vac operation)
Resuspend pellet in TE buffer or H_2O (20-50µl)

TE buffer stands for Tris/ EDTA. The EDTA protects the DNA from enzymatic degradation. To "resuspend" the pellet, vortex 5 seconds and then flick with your finger. If the pellet is stubborn, you may want to gently pipet it up and down.

TE buffer (10mM Tris, 1mM EDTA)
2ml 1M Tris
400µl .5M EDTA
in 200ml H2O.

pGEM-T Easy Vector:

Once you purify the PCR products, you can insert them into a plasmid vector. We use Promega's pGEM-T Easy Vector. It consists of 10x buffer, ligase, and the vector itself. A control tube is also included to test the efficiency of the vector. We usually do not use the control. The components may be in the freezer in aliquots. This is because the stuff does not like to be frozen and thawed repeatedly. Do not leave the tubes on the bench for any period of time. Take them from the freezer, remove how much you need and replace the tubes immediately.

Inserting DNA into a Vector
Combine the following in a small epindorf tube
2µl T4 DNA Ligase 10X Buffer
1µl pGEM-T Easy Vector
1µl T4 DNA Ligase
Add 6µl insert DNA
Keep at 4° overnight
control (optional): Use 6µl H_2O in place of insert DNA

Transforming into Competent Cells:

Once you have your insert in a vector, you need to heat shock the vector into competent E.coli cells (transformation). Make sure you use competent cells with the endA 1 mutation.

Transformation Protocol
Heat vector to 65° for 15 minutes
Thaw deep frozen competent cells in an ice bucket.
Gently add vector to 300µl competent cells.
Soak at 37° for 3 minutes
Add 1ml LB (no AMP)
Shake for 1 hour
centrifuge and resuspend in 80µl LB
Add 5µl to one AMP/X-Gal plate, 75µl to another
(See Plating and 2ml Cultures, page 15)
Incubate at 37° overnight.

Plating and 2ml Cultures:

Bacterial colonies are first grown on agar plates and select colonies are then transferred to 2ml cultures of liquid medium. Label the bottom of plates with the name of the sample, the date, and your room number. Grow plates overnight at 37° in the warm fridge (room 410)

Blue/White AMP Plates (~ 20 plates)
5g tryptone
2.5g yeast
2.5g NaCl
7.5g agar
in 500ml H_2O
Put aluminum foil over the top of the flask and autoclave
Cool to 60° then add 1ml AMP (50mg/ml)
Pour into plates until the bottom is just covered
let the plates harden (~20 minutes)
add 10µl/plate of 0.1M IPTG (0.238g in 10ml H_2O)
and 80µl/plate 2% X-Gal (0.8g X-Gal in 40ml DMSO)

To make 2ml cultures:
Add 100µl AMP (50mg/ml) to 50 ml Terrific Broth

Terrific broth[1] (1L)
12g tryptone
24g yeast
4ml pure glycerol
2.31g $KH_2(PO_4)$
12.54g K_2HPO_4
in 1L H_2O
Distribute 250ml into 8 flasks
Autoclave (put foil over top of flasks)

After you add AMP, make at least 20 2ml cultures in sterile test tubes.
Remove single white colonies from the plates with a flamed wire loop or sterile toothpick. (Remember blue colonies indicate plasmids with no inserts.)
Inoculate the 2ml cultures
Put them in the 37° shaker (room 410) overnight.
When the 2ml cultures come out of the shaker, they should appear cloudy. A cloudy solution indicates bacteria have grown.

This procedure is sometimes called a mini-prep or SPP (small plasmid prep) as opposed to large-scale preps which are useful for isolating large quantities of plasmid DNA. Large-scale preps require 500ml bacteria cultures, but 1-5ml cultures are sufficient for mini-preps.

Alkaline Lysis Mini-Prep

Pour your 2ml cultures into large epindorf tubes
Save the excess cultures in case you need to grow more
Spin the cultures down in the ultracentrifuge (room410) for 5- 10min
Pour off the supernatants
Bacterial pellets should be visible.
Resuspend the pellets in 100µl of Solution 1

Solution 1
18µl 50% glucose
20µl 0.5M EDTA
25µl 1M Tris
4mg lysozyme
in 1ml H_2O
keep in the fridge

Mix and wait 5min. The solutions should become gelatinous
Add 200µl room-temp Solution 2

Solution 2
75µl SDS
1350µl H_2O
75µl 4M NaOH
keep at room temp

Mix by inverting and wait 5min
add 150µl Solution 3

Solution 3
(3M KoAc pH 5)
29.44g KoAc
11.5ml glacial acetic acid
in 100ml H_2O
keep in the fridge

Wait 5min. Flocculant white precipitates should form
Spin in the ultra centrifuge for 10min
Transfer the supernatants to fresh epindorf tubes containing 1ml 100% ethanol
Spin down for 10 minutes in the ultracentrifuge
Pour off the supernatant and dry the tubes upside down on a paper towel
Wash pellet with 70% ethanol:

Add 500µl 70% EtOH and vortex briefly to dislodge pellet
Spin down for 10 min in the ultracentrifuge

Pour off ethanol without pouring away the pellet
Use a speed-vac if possible to dry the pellet (See appendix).
Resuspend your DNA in 20µl of TE with RNAse (final concentration of 20µg/ml)
Transfer 5µl of the finished plasmid to a small epindorf tube for enzyme digestion

Enzyme Digestions:

Once you get your plasmid it is time to test and see if you were successful in cloning your band. This is accomplished with an enzyme digestion. The pGEM-T Easy vector we use has an EcoR1 site on both sides of the insert. Therefore a digest with EcoR1 will cut our insert out again. If the results are not satisfactory, or if you have no EcoR1, you can use a combination of enzymes, choosing two that cut one on each side of the insert. An alternate method is the PCR screen for inserts (See appendix).

Enzyme Digestion
13µl H2O
2µl buffer
5µl DNA
1µl enzyme
(If you want a no-enzyme control use 14µl H2O)
Spin down briefly
Incubate at 37° for 2-4 hours (water bath)
Add 2µl loading buffer
Run on 100ml gel

Loading Buffer
(30% glycerol, .25% bromophenol blue)
0.3g glycerol
0.0025g bromophenol blue
in 1ml H_2O

When you look at the digestion on the light box, you should see bright bands near the wells, which are the cut plasmids. With hope, some of your wells will also have bands the same size as your original insert. If you do see the insert you can take one or more of those samples to be sequenced. The DNA should be fairly bright on the gel. If it is not, you may have to get an absorbance reading on your sample at 260nm. Ideally, DNA should be at a concentration of 250ng/µl for sequencing. An OD of 1.000 is equal to 50ng./µl, so if you make a dilution of 10µl/ml, you should get a reading of 0.0500. If your reading is higher, you can add H_2O. If your reading is between 0.0250 and 0.0500, you can still get it sequenced but just put a note on the request sheet. If your reading is lower than 0.0250, you will need to ethanol precipitate the DNA and resuspend in a lower volume of H_2O (See Ethanol Precipitation- page 13).

Appendix:

SOB broth (1L)
20g tryptone
5g yeast
0.5g NaCl
2.5ml 1M KCl
0,2ml 5M NaOH (brings pH to 7.0)
in 1000ml H_2O
Autoclave and allow to cool
Just before use: add 5ml $MgCl_2$
and 2ml AMP (50 mg/ml)

CTAB extraction
If you are doing more than one plant, be careful not to contaminate one with the other. (Clean the razors blade, change pipet tips between solutions etc.)
Cut off up to 3g of leaf tissue with a clean razor blade (wipe with 70% EtOH)
Freeze with liquid nitrogen (from the store room) and grind to a powder with an autoclaved mortar and pestle.
Transfer the leaf matter to a 50ml centrifuge tube
Add 15ml extraction buffer

Extraction Buffer
(0.1M Tris-Cl pH 8/ 0.05M EDTA/ 0.5M NaCl/ 0.01M ß-mercaptoethanol)
1.21g Tris
2.92ml 1M HCl
1.86g EDTA
2.92g NaCl
in 100ml H_2O
add 7.8µl/ml ß-mercaptoethanol just before use
Use the hood when handling the ß-mercaptoethanol. It has a foul odor.

Add 1ml 20% SDS
Incubate at 65º for 10 minutes
Add 5ml 5M potassium acetate (KOAc)
Shake vigorously and keep on ice for 20 minutes

Spin tubes in the Sorvall RC 5B Plus centrifuge at 14,500 rpm for 13 minutes
(Use the smaller rotor (SS34))
Filter supernatant into 10ml of isopropanol
Keep in the freezer for 30 minutes
Pellet the DNA at 13,000 rpm for 20 minutes
Pour away the supernatant and dry the tubes upside down on a paper towel
Resuspend the DNA in 700µl of TE buffer (heat to 65º if necessary)
Transfer to an epindorf tube and spin down to remove any insolubles
Move the supernatant to a new tube and add 50µl 3M NaOAc
and 100µl 1% CTAB (Cetyl Trimethyl Ammonium Bromide)
Pellet the solution for 1 minute in the microcentrifuge
discard the supernatant and wash the pellet in 70% ethanol
Resuspend pellet in 400µl of TE buffer
Ethanol precipitate the DNA with 50µl 3M NaOAc and 1ml 100% ethanol
(only freeze 5- 10 minutes and spin 10 - 15 minutes)
Repeat two more times to get rid of all residual CTAB
Wash with 70% ethanol
(add 500µl 70% EtOH and vortex briefly to dislodge pellet
Spin down for 10 min in the ultracentrifuge)
Dry in speed vac if possible
resuspend the DNA in 100µl TE
Do a spec reading on the DNA to determine the concentration
Dilute as necessary to 5ng/µl.

Techniques to improve Mini-preps.

8000 Precipitation:

This purification technique removes RNA and other goo from your DNA
Add an equal volume of a 13% PEG 8000, solution to your plasmid DNA:

13% PEG 8000
4ml 1M NaCl
1.3g PEG 8000
in 10ml H_2O

Mix and spin in the ultracentrifuge for 10min
Resuspend the pellet in TE buffer.

Extraction:

Phenol extraction is a purification technique to remove protein that should only be used under extreme circumstances. Phenol is a dangerous chemical that can cause severe burns. Always use gloves and safety glasses when handling phenol.

Add to your plasmid DNA an equal volume of phenol
mix and centrifuge, transfer top layer to a fresh tube
add 1/2 volume phenol and 1/2 volume chloroform with 4% isoamyl alcohol
mix and centrifuge, transfer top layer to a fresh tube
Add equal volume chloroform
mix and centrifuge, transfer top layer to a fresh tube
ethanol precipitate the remaining liquid.

PCR Screen for Inserts:

An alternative method for screening for inserts is to do a PCR reaction using the pGEM-T Easy Vector's primers (T7 and SP6). This method is recommended if you are getting unclear results with your enzyme digestions or want to cut out unnecessary mini-preps.

Dilute 10µl of your 2ml bacterial culture and dilute it in 1ml of H_2O
Use 2µl of this dilution as your template DNA
Use the pGEM-T Easy Vector's T7 and SP6 primers
Use Taq and buffer as usual
Change the annealing temperature to 50°

Speed Vac Operation:

One Savant Speed Vac SVC 100 is located in the sequencing lab (room 303) and another is in Dr. Altchuler's lab (room 323). They allow for thorough drying of samples by spinning them in a vacuum.

Before operating, make sure the Condensation Trap RT 100 is on.
Open chamber lid. If it won't open, the vacuum is on; turn the red bleed arrow on the air hose so it faces the chamber.
Load epindorf tubes opposite one another with the lids open
Close chamber lid
Turn concentrator on. Tubes should start spinning

Turn the bleed arrow 90° until it is facing you
Turn on the pump
Wait 10 minutes
Turn bleed arrow to chamber
After hissing stops, turn concentrator off and remove tubes
If they are completely dry, turn bleed arrow towards the pump
and immediately turn pump off

References

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Ausubel, Brent, Kingston, Moore, Seidman, Smith, and Struhl, Current Protocols in Molecular Biology, Wiley Interscience, 1989

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[1]Adapted from Tartof and Hobb's recipe in Molecular Cloning, A.2