

PCR Cloning of NBS-containing Sequences from Grape

I. Objective

The objective of this lab unit is to clone partial genomic sequences corresponding to the NBS(nucleotide binding site) sequences in grapes.

II. Introduction

Most commercially grown grape cultivars are *Vitis vinifera* spp that are susceptible to grape pathogens. In contrast, the Asian grape, *Vitis Amurensis*, has strong resistance to anthracnose and ripe rot, and moderate resistance to downy mildew and powdery mildew. The variation is partially due to the presence of disease resistance (R) genes in tolerant/resistant grapes. Proteins encoded by R genes have been cloned from a wide range of plant species. Many of them are found to encode nucleotide-binding site and leucine-rich repeat (NBS-LRR) proteins which are involved in detecting diverse pathogens, i.e. bacteria, viruses, fungi, nematodes, insects and oomycetes. NBS-LRR encoding sequences are identified through gene cloning, genome sequencing projects, and PCR amplification. Polymerase chain reaction (PCR) using degenerate primers is highly sensitive in isolating conserved sequences and it would be effective in obtaining potential *nbs-lrr* disease-resistance genes from genomic DNAs. Conserved regions within the NBS domain are used to design degenerate primers to PCR-amplify the corresponding DNA sequences. The clones can be developed into markers for discriminating resistant and susceptible grape cultivars and are useful in breeding programs.

To clone grape *nbs*-containing sequences, we will first isolate genomic DNA from grape leaves. The essence of genomic DNA extraction is to purify DNA from cellular material in a manner that prevents DNA degradation. Procedures for DNA extraction from plant tissue vary depending on the material used. Basically, all procedures involve mechanical breakdown of the cell wall and membranes to allow access to nuclear material without degrading DNA in the process. For this, an initial grinding stage with liquid nitrogen is usually employed to break down cell wall and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the tissue is ground, it can then be resuspended in a proper buffer, such as CTAB/SDS. To purify DNA, unwanted insoluble materials are removed through centrifugation while soluble proteins and other material are separated through further purification steps. DNA must then be precipitated from the aqueous phase and washed to remove contaminating salts. The purified DNA is then resuspended and stored in TE buffer or sterile distilled water. The quality of the extracted DNA can be checked by running the DNA sample on an agarose gel stained with ethidium bromide that is then visualized under UV light.

After genomic DNA isolation and purification, a UV spectrophotometer (NanoDrop) is used to determine the quantity and purity of the genomic DNA obtained. The absorbances (O.D.) at various wavelengths indicate a variety of impurity in the DNA

sample (Table 1). The concentration of the DNA sample can also be estimated using the equation shown below.

Table 1 Peak absorbance of contaminated substances in a DNA sample

Peak absorbance (nm)	Substance absorbing at that wavelength
230	EDTA, polysaccharides, ethanol
260	DNA, RNA
270	Phenol
280	Proteins
320	Cell debris scatters light

$$\text{Concentration of DNA } (\mu\text{g/ml}) = (A_{260} - A_{320}) \times \frac{50 \mu\text{g/ml}}{1 \text{ absorbance unit}} \times \text{dilution.}$$

1 A₂₆₀ = 33 μg/ml ssDNA; 50 μg/ml dsDNA; 40 μg/ml RNA

The isolated genomic DNA is then amplified by PCR using degenerate primers (LM 638, LM 637) corresponding to a conserved region within NBS domain (Figure 1).

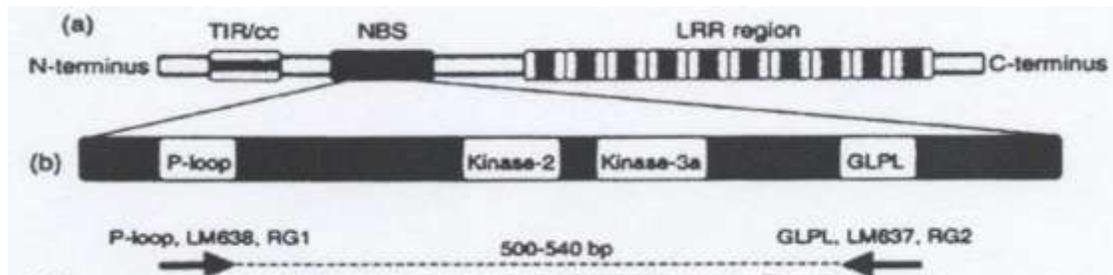
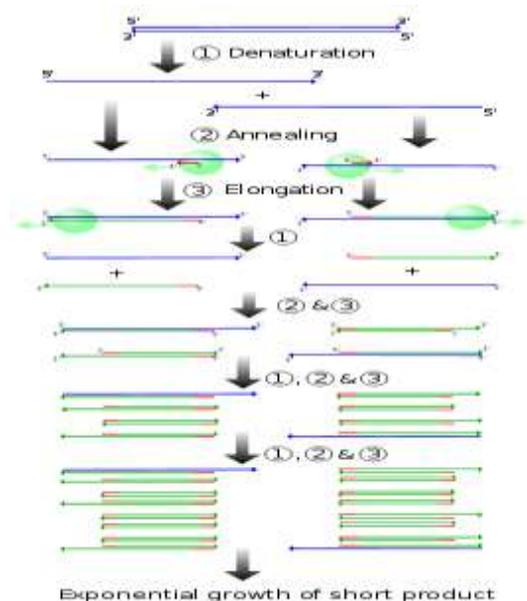


Figure 1 (a) Model of the NBS-LRR type of R-genes with a TIR or a coiled-coil (cc) N-terminal domain. (b) The most conserved regions within the NBS and the degenerate primers, LM 638 (5'-GGIGGIGTIGGIAAIACIAC-3') and LM 637 (5'-A(A/G)IGCTA(A/G)IGGIA(A/G)ICC-3'). I: inosine

For PCR, DNA template is incubated in the presence of gene specific primers (or degenerate primers in our case), dNTPs, and Taq DNA polymerase with an appropriate buffer. The reaction is cycled at 94°C, ~55°C, and 72°C for 25-35 times. The 94°C step denatures the double helix (denature), the ~55°C step allows annealing of most primers (anneal), and the 72°C step is the optimal temperature for most Taq polymerase (extension). The amplification steps are shown in the figure on the right.



The PCR-amplified products are then cloned into a plasmid vector. The steps in gene cloning include (1) isolating/ purifying DNA, (2) ligating the DNA into a cloning vector, i.e. a plasmid, (3) transforming a host cell, i.e. *E. coli*, with the recombinant DNA (vector with the DNA insert), (4) screening for bacterial colonies harboring the recombinant DNA or producing the appropriate protein product. For step (1), the PCR products are first purified by agarose gel electrophoresis followed by cutting and extracting the target DNA band from the gel (week 2). The purified DNA is ligated into a cloning vector that is then transformed into *E. coli* and screened for bacterial colonies with the recombinant DNA (steps 2-4) (week 3). Finally, large quantity of rDNAs are obtained by isolating plasmid DNAs from an overnight culture of a single transformed bacterial colony and rDNAs are ready for subsequent restriction mapping and sequence analysis (Unit II).

Week 1

Plant genomic DNA Isolation and Quantification; PCR using degenerate primers

Objective

The objective of this lab exercise is to isolate genomic DNA from grape leaves and amplify the genomic DNA through PCR using degenerate primers corresponding to a conserved region within the NBS domain of NBS-LRR proteins.

Plant genomic DNA Isolation

Things to do before starting

- Buffer AP1 and Buffer AP3/E concentrate may form precipitates upon storage. If necessary, warm to 65°C to redissolve (before adding ethanol to Buffer AP3/E).
 - Do not heat Buffer AP3/E after ethanol has been added.
 - Buffer AW and Buffer AP3/E are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- 1. Grind 100 mg (or less) plant sample under liquid nitrogen to fine powder and transfer to a new tube.**

Do not allow the sample to thaw, and continue immediately to step 2. (Instructor does this step)
 - 2. Add 400 µl of AP1 and 4 µl of RNase A solution (100 mg/ml) to the tissue powder, vortex vigorously, incubate the mixture at 65C for 10 minutes. Invert 2-3 times during 65C incubation.**
 - Do not mix AP1 Buffer and RNase A prior to use.

- No tissue clumps should be visible. Vortex or pipet further to remove any clumps. Clumps of tissue will not lyse properly and will therefore result in a lower yield of DNA. In rare cases, where clumps cannot be removed by pipetting and vortexing, a disposable micropestle may be used.

This step lyses the cells.

3. Add 130 μ l of AP2 Buffer to the lysate, vortex, and incubate on ice for 5 minutes.

This step precipitates detergent, proteins, and polysaccharides.

Recommended: Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm).

- Some plant materials can generate very viscous lysates and large amounts of precipitates during this step. This can result in shearing of the DNA in the next step. In this case, optimal results are obtained if the majority of these precipitates are removed by centrifugation for 5 min at 20,000 x g (14,000 rpm). After centrifugation, apply supernatant to QIAshredder Mini spin column.
- 4. Pipet the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at 20,000 x g (14,000 rpm).**
- It may be necessary to cut the end off the pipet tip to apply the lysate to the QIAshredder Mini spin column. The QIAshredder Mini spin column removes most precipitates and cell debris, but a small amount will pass through and form a pellet in the collection tube. **Be careful not to disturb this pellet in the next step.**
- 5. Transfer the flow-through fraction from step 4 into a new tube (not supplied) without disturbing the cell-debris pellet.**
- Typically 450 μ l of lysate is recovered. For some plant species less lysate is recovered. In this case, determine the volume for the next step.
- 6. Add 1.5 volumes of Buffer AP3/E to the cleared lysate, and mix by pipetting.**
- For example, to 450 μ l lysate, add 675 μ l Buffer AP3/E. Reduce the amount of Buffer AP3/E accordingly if the volume of lysate is smaller. A precipitate may form after the addition of Buffer AP3/E, but this will not affect the procedure.
- Note:** It is important to pipet Buffer AP3/E directly onto the cleared lysate and to mix immediately.
- 7. Pipet 650 μ l of the mixture from step 6, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube (supplied). Centrifuge for 1 min at \geq**

6000 x g (corresponds to ≥ 8000 rpm for most microcentrifuges), and discard the flow-through.*

Reuse the collection tube in the next step.

8. Repeat step 7 with remaining sample. Discard flow-through and collection tube.

Steps 6- 8 precipitate and bind the DNA into the column.

9. Place the DNeasy Mini spin column into a new 2 ml collection tube (supplied), add 500 μ l Buffer AW, and centrifuge for 1 min at ≥ 6000 x g (≥ 8000 rpm). Discard the flow-through and reuse the collection tube in the next step.

Note: Ensure that ethanol is added to Buffer AW.

This step gets rid of excess salt.

10. Add 500 μ l Buffer AW to the DNeasy Mini spin column, and centrifuge for 1 min at ≥ 6000 x g (≥ 8000 rpm).

11. Centrifuge at 20,000 x g (14,000 rpm) for 2 minutes to remove traces of WS Buffer.

Note: Following the centrifugation, remove the DNeasy Mini spin column from the collection tube carefully so the column does not come into contact with the flowthrough, as this will result in carryover of ethanol.

- It is important to dry the membrane of the DNeasy Mini spin column since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during elution. Discard flow-through and collection tube.
- After washing with Buffer AW, the DNeasy Mini spin column membrane is usually only slightly colored. In the rare case that the membrane remains significantly colored after washing with Buffer AW, reduce the amount of starting material in future preps or add an additional wash with 500 μ l ethanol (96–100%) and then centrifuge for 2 min at 20,000 x g (14,000 rpm) to dry the membrane.

12. Transfer the DNeasy Mini spin column to a 1.5 ml microcentrifuge tube (not supplied), and pipet 100 μ l Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature (15–25°C), and then centrifuge for 1 min at ≥ 6000 x g (≥ 8000 rpm) to elute.

- Elution with 50 μ l (instead of 100 μ l) increases the final DNA concentration in the eluate significantly, but also reduces overall DNA yield. If larger amounts of DNA (>20 μ g) are loaded, eluting with 200 μ l (instead of 100 μ l) increases yield.

Note: More than 200 μ l should not be eluted into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

13. Label the tube with your section and group no. and store DNA at -20C.

DNA quantification using NanoDrop

1. Log In (Research)
2. Click "ND-1000 3.3" and then "Nucleic Acid"
3. Clean up the pedestals with 2 μ l of RO water and wipe off the water with Kimp wipes
4. Load 2 μ l RO water, close the pedestals and click "DNA" and "OK" to initialize the instrument
5. Wipe off the water with Kim wipes, add 2 μ l of TE/ EB, close the pedestals, and click "Blank" to zero the instrument
6. Wipe off the TE/ EB with Kim wipes, add 2 μ l of your DNA sample, close the pedestals, and click "Measurement" to read the O.D. ratio and concentration (ng/ μ l) of your sample.

PCR (polymerase chain reaction)

Polymerase Chain Reaction (PCR), invented by **Kary B. Mullis**, at the Cetus Corporation, who was awarded the 1993 Nobel Prize for chemistry for PCR.

1. Set up **one** 0.5 ml thin-walled PCR tube containing the following reaction mix on the right.

Instructor will make up the 40 μ l of PCR reaction mix and you will add 10 μ l H₂O w/ 100 ng of genomic DNA

1. Add your plant genomic DNA into the tube last. (**Use a filter tip**)
2. Close the cap tightly and amplify the samples using an automated thermal cycler.

PCR reaction mix: either primer

RO water	31.5 μl
10 X buffer (with MgCl₂)	5 μl
Mixture	3 μl
dNTPs: 1 μ l of 10 mM each	
LM 638 or LM 637: 2 μ l (25 pmol)	
Taq polymerase	1 μl (2.5 U)
Plant Genomic DNA	10 μl (100 ng)
Total	50 μl

PCR reaction mix: both primers

RO water	31.5 μl
10 X buffer (with MgCl₂)	5 μl
Mixture	3 μl
dNTPs: 1 μ l of 10 mM each	
LM 638: 1 μ l (25 pmol)	
LM 637: 1 μ l (25 pmol)	
Taq polymerase	1 μl (2.5 U)
Plant Genomic DNA	10 μl (100 ng)
Total	50 μl

PCR Cycle

94 °C for 2 minute (1 cycle)

94 °C for 30 seconds, 45 °C for 30 seconds, 65°C for 1 minute (35 cycles)

Week 2

Agarose Gel Electrophoresis; DNA Extraction from Gel and Quantification

Objective

The objective of this lab exercise is to purify the PCR product by separating the PCR-amplified DNA fragments in an agarose gel and then extracting the target band from the gel.

Introduction

Agarose is a neutral linear polysaccharide purified from agar-agar of red algae. It is made of D-galactose and 3, 6-anhydro-L-galactose linked by glycosidic bonds. Agarose is soluble in hot water. When the dissolved agarose solution cools, a matrix forms (a gel) through the crosslinking of agarose polymer chains by hydrogen bonds. The X-ray diffraction shows that the agarose molecules have a double-helical structure. The higher the agarose concentration in the gel, the more fibers per cm^3 are (smaller pore sizes). Also, the presence of salt increases the pore sizes of the gel.

DNA is a repeating polymer and thus has a constant charge/mass ratio. Gels of a specific pore size can be used to separate DNA molecules of different sizes. In addition to agarose concentration, the mobility of DNA is affected by its size, conformation, applied current/voltage, temperature and salt. Increasing ionic strength decreases mobility (extra ions shield DNA from the electric field). For this lab exercise, we will separate the PCR-amplified DNA fragments obtained from last week's lab by agarose gel electrophoresis. After gel electrophoresis, we will extract the target DNA band from gel using Qiagen's MinElute Gel Extraction Kit and estimate the quantity and purity of the isolated DNA using NanoDrop.

Agarose Gel Electrophoresis

Note: *Bring a USB drive, a pair of safety glasses and do the first 4 steps 40 mins before class.*

1. Heat 1 g of agarose plus 100 ml of 1 X TAE (1 ml of 50 X TAE) in a 250 ml flask until agarose is completely melted.
2. Allow to cool to about 60°C with frequent swirling.
3. Add 3 μl ethidium bromide and mix well.
4. Pour the agarose solution into a pre-sealed gel tray, insert the two sample combs and allow it to set.
5. Place the gel tray in the electrophoresis box and add 300 ml of 1 X TAE buffer.
6. Add 12.5 μl of DNA loading buffer into your PCR tube (Instructor did this already).
7. For the top gel, load 20 μl of PCR product amplified with each individual primers at the first two lanes followed by 5 μl of DNA molecular weight size marker
8. Load 20 μl of your PCR samples into the next well.

9. Repeat the same procedure for the bottom gel.
10. Electrophorese samples at constant voltage, ~100 V/gel, until the bromophenol blue is approximately 3/4 of the distance through the gel (~ 60 mins).
11. Visualize the DNA fragments under Gel Logic 200 (See Imaging a Gel with Gel Logic 200), photograph and isolate the fragment from the gel (See MinElute Gel Extraction Kit Protocol).

Imaging a Gel with Gel Logic 200

1. Turn on computer and log in (username: Research, password: research)
2. Open door of imager
3. Place gel at center of platform; smooth out bubbles
4. Close imager door; turn dial to Trans UV
5. Click KODAK MI icon on desktop
6. Click Capture Gel 100...
7. Set Custom Settings to Ethidium Bromide
8. Click Preview
9. Open imager door and adjust gel position so it is centered on the imaging screen
 - * If the image is very faint, click + coarse adjustment to increase exposure time
 - * Zoom in/out using the middle dial on the imager camera
 - * Focus / sharpen the image using the lower dial on the imager camera
10. When gel image is good, click Capture
13. Click File Export Data → Image Save in: mol tech (2011) folder
14. Also save on your flashdrive (do not handle flashdrive with gloves)
15. Open imager door; remove gel; wipe off platform with a Kimwipe
16. Turn imager dial to Off
17. Shut down the computer

MinElute Gel Extraction Kit Protocol

This protocol is designed to extract and purify DNA of 70 bp to 4 kb from standard or low-melt agarose gels in TAE/TBE buffer resulting in high end-concentrations of DNA. ≤400 mg agarose can be processed per column.

Important points before starting

- The yellow color of Buffer QG indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at ≥10,000 x g in a table-top microcentrifuge at room temperature.

Procedure

- 1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**
Minimize the size of the gel slice by removing extra agarose.
- 2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg or approximately 100 µl).**
For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per spin column is 400 mg; for gel slices >400 mg use > one MinElute column.

- 3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**
Solubilize agarose completely. For >2% gels, increase incubation time.
- 4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).**
If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow. The adsorption of DNA to the membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.
- 5. Add 1 gel volume of isopropanol to the sample and mix by inverting the tube several times.**
For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. Do not centrifuge the sample at this stage.
Place a MinElute column in a provided 2 ml collection tube in a suitable rack.
- 6. To bind DNA, apply the sample to the MinElute column, and centrifuge for 2 min.**
For maximum recovery, transfer all traces of sample to the column. The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
- 7. Discard the flow-through and place the MinElute column back in the same collection tube.**
- 8. Add 500 µl of Buffer QG to the spin column and centrifuge for 1 min.**
- 9. Discard the flow-through and place the MinElute column back in the same collection tube.**
- 10. To wash, add 750 µl of Buffer PE to the MinElute column and centrifuge for 1 min.**
If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
- 11. Discard the flow-through and centrifuge the MinElute column for another 1 min at ≥10,000 x g.**
Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
- 12. Place the MinElute column into a clean 1.5 ml microcentrifuge tube.**
- 13. To elute DNA, add 20 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.**
Ensure that the elution buffer is dispensed directly onto the center of the membrane for complete elution of bound DNA. The average eluate volume is 9 µl from 10 µl elution buffer volume.
Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Week 3

DNA ligation and bacterial transformation using StrataClone PCR Cloning Kit

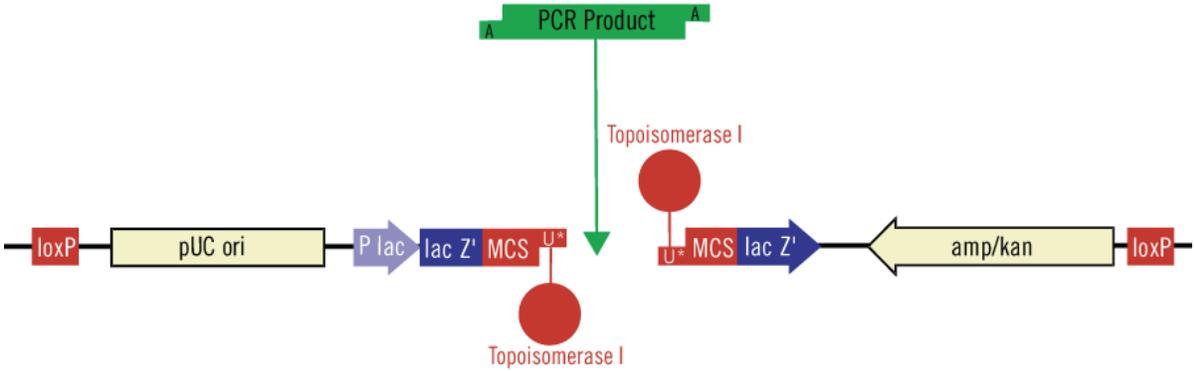
Objective

The objective of this lab exercise is to clone the purified PCR product into StrataClone PCR cloning vector pSC-A-amp/kan and then transform the plasmid into competent bacterial cells.

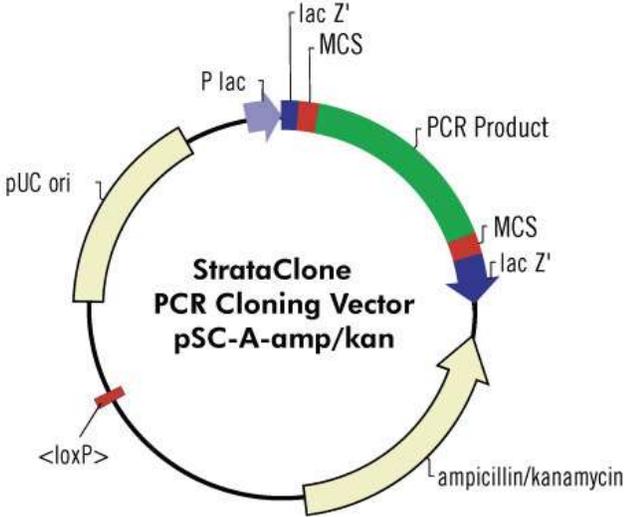
Overview of StrataClone PCR Cloning

The cloning combined activities of topoisomerase I from *Vaccinia* virus and Cre recombinase from bacteriophage P1. *In vivo*, DNA topoisomerase I assists in DNA replication by relaxing and rejoining DNA strands. Topoisomerase I cleaves the phosphodiester backbone of a DNA strand after the sequence 5'-CCCTT, forming a covalent DNA-enzyme intermediate which conserves bond energy to be used for religating the cleaved DNA back to the original strand. Once the covalent DNA-enzyme intermediate is formed, the religation reaction can also occur with a heterologous DNA acceptor. The Cre recombinase enzyme catalyzes recombination between two *loxP* recognition sequences. The StrataClone PCR cloning vector mix contains two DNA arms, each charged with topoisomerase I on one end and containing a *loxP* recognition sequence on the other end. The topoisomerase-charged ends have a modified uridine (U*) overhang. *Taq*-amplified PCR products, which contain 3'-adenosine overhangs, are efficiently ligated to these vector arms in a 5-minute ligation reaction, through A-U* base-pairing followed by topoisomerase I-mediated strand ligation. The resulting linear molecule (vector armori-PCR product-vector armamp/kan) is then transformed, with no clean-up steps required, into a competent cell line engineered to transiently express Cre recombinase. Cre-mediated recombination between the vector *loxP* sites creates a circular DNA molecule (pSC-A-amp/kan, see Figure 2) that is proficient for replication in cells growing on media containing ampicillin or kanamycin. The resulting pSC-A-amp/kan vector product includes a *lacZ* α -complementation cassette for blue-white screening. (See the figure below)

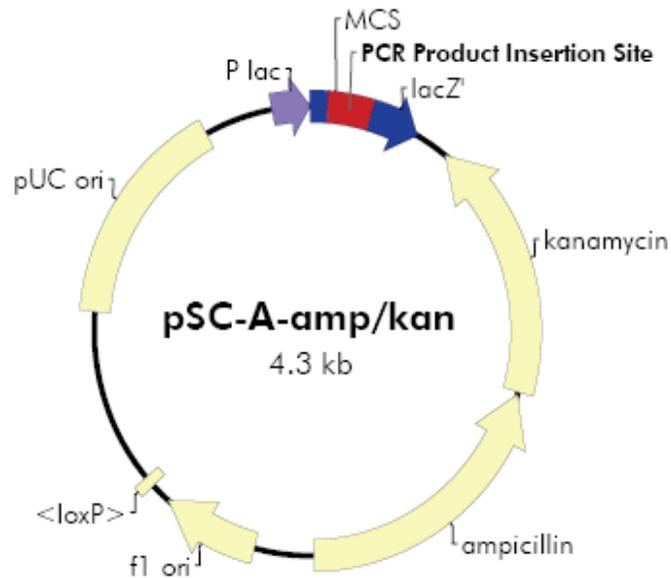
**Incubate PCR product with
Topoisomerase I-charged
vector arms (5 minutes)**



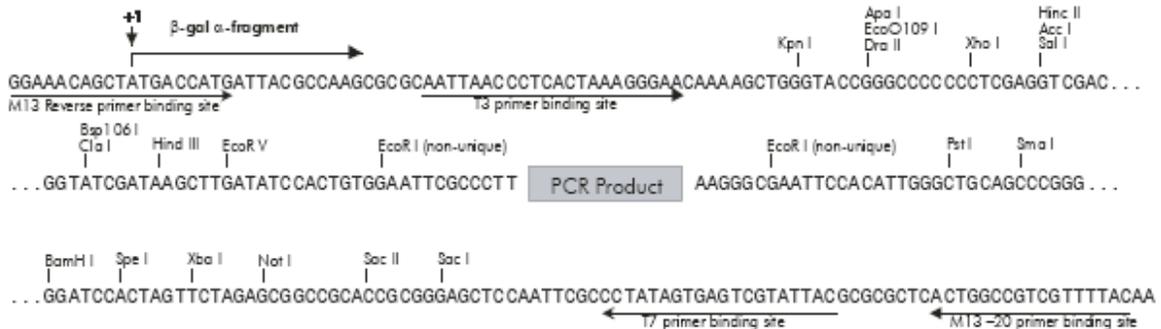
**Transform StrataClone competent cells
expressing Cre recombinase**



Map for the StrataClone PCR Cloning Vector pSC-A-amp/kan



pSC-A-amp/kan PCR Cloning Vector PCR Product Insertion Site Region (sequence shown 4261–4270, 1–250)



Feature	Nucleotide Position
β -galactosidase α -fragment coding sequence (<i>lacZ'</i>)	1–352
Multiple cloning site (MCS)	57–195
PCR product insertion site	123
Kanamycin resistance ORF	463–1254
ampicillin resistance (<i>bla</i>) ORF	1266–2123
f1 origin of ss-DNA replication	2315–2621
<loxP> (mutant <i>loxP</i> -derived sequence <i>lox66/71</i> ; nonfunctional in Cre-mediated recombination)	2688–2721
pUC origin of replication	3262–3929
<i>lac</i> promoter	4151–4270

FIGURE 2 StrataClone PCR cloning vector pSC-A-amp/kan. The circular map shown represents the product of topoisomerase I-mediated ligation of the supplied vector arms to a PCR product of interest followed by Cre-mediated recombination. The complete sequence and list of restriction sites are available at www.stratagene.com.

PCR CLONING PROTOCOL

Note *StrataClone vector arms purchased prior to 2/1/2008 do not carry the kanamycin resistance marker. If planning to use kanamycin selection, ensure that the cloning reaction is performed using StrataClone Vector Mix amp/kan (as listed on the tube label).*

Preparing the PCR Product

1. Prepare insert DNA by PCR using *Taq* DNA polymerase or an enzyme blend qualified for PCR cloning applications.

Note *Taq DNA polymerase is required for the addition of 3' adenine residues to the PCR product. If PCR was performed using a proofreading DNA polymerase, see Appendix II for a protocol for adding 3' -A overhangs after the PCR reaction is complete.*

2. Analyze an aliquot of the PCR reaction on an agarose gel to verify production of the expected fragment.
3. If the fragment to be cloned is **< 3 kb and gel analysis confirms robust, specific** amplification, prepare a **1:10 dilution of the PCR reaction in dH₂O**. For larger or poorly amplified fragments, omit the dilution step.

Note *If multiple PCR products are observed on the gel, or when cloning very large PCR products, gel isolate the desired PCR product prior to performing the ligation reaction. See Appendix I for a gel-isolation protocol. For a gel-isolated PCR product recovered in 50 µl, add 2 µl (undiluted) of the purified PCR product to the ligation reaction below.*

Ligating the Insert

4. Prepare the ligation reaction mixture by combining (in order) the following components:

3 µl StrataClone Cloning Buffer

2 µl of PCR product (5-50 ng, typically a 1:10 dilution of a robust PCR reaction) or 2 µl of StrataClone Control Insert

1 µl StrataClone Vector Mix amp/kan

5. Mix gently by repeated pipetting, and then incubate the ligation reaction at room temperature for 5 minutes. When the incubation is complete, place the reaction on ice.

Note *The cloning reaction may be stored at -20°C for later processing.*

Transforming the Competent Cells

6. Thaw one tube of StrataClone SoloPack competent cells on ice for each ligation reaction.

Note *It is critical to use the provided StrataClone SoloPack competent cells, expressing Cre recombinase, for this protocol. Do not substitute with another strain.*

7. Add 2 μ l of the cloning reaction mixture to the tube of thawed competent cells. Mix gently (do not mix by repeated pipetting).

Note *For large PCR products, up to 2 μ l of the cloning reaction mixture may be added to the transformation reaction.*

If desired, test transformation efficiency of the competent cells by transforming a separate tube of competent cells with 10 pg of pUC18 control DNA. Prior to use, dilute the pUC18 DNA provided 1:10 in dH₂O, and then add 1 μ l of the dilution to the tube of competent cells.

8. Incubate the transformation mixture on ice for 20 minutes. During the incubation period, pre-warm LB medium to 42°C.

9. Heat-shock the transformation mixture at 42°C for 45 seconds.

10. Incubate the transformation mixture on ice for 2 minutes.

11. Add 250 μ l of pre-warmed LB medium to the transformation reaction mixture. Allow the competent cells to recover for at least 1 hour at 37°C with agitation. (Lay the tube of cells on the shaker horizontally for better aeration.)

Note *When selecting transformants on kanamycin plates, increasing the recovery period to 1.5-2 hours will increase the number of transformants obtained.*

12. During the outgrowth period, prepare LB-ampicillin plates or LB-kanamycin plates[§] for blue-white color screening by spreading 40 μ l of 2% X-gal on each plate.

13. Plate 10 and 100 μ l of the transformation mixture on the color- screening plates. Incubate the plates overnight at 37°C.

Notes *For the Control insert cloning reaction, plate 10 μ l of the transformation mixture on LB-ampicillin plates.*

For the pUC18 control transformation, plate 30 μ l of the transformation mixture on LB-ampicillin plates.

When spreading <50 μ l of transformation mixture, pipette the cells into a 50- μ l pool of LB medium before spreading.

*See Preparation of Media and Reagents.

Analyzing the Transformants

14. Pick white or light blue colonies for plasmid DNA analysis. Do not pick dark blue colonies.

Notes *Colonies harboring plasmids containing typical PCR product inserts are expected to be white. After prolonged incubation, some of the insert-containing colonies may appear light blue. If the insert contains an in-frame start codon proximal to a ribosome binding site, a functional LacZ' a-fragment fusion protein may be produced. This typically results in blue or light blue colonies for one insert orientation. If large numbers of blue colonies are obtained, analyze the DNA from a selection of these colonies for the presence of the insert.*

15. Prepare miniprep DNA from the selected colonies using standard protocols. Perform restriction digestion analysis of the miniprep DNA to identify colonies harboring the desired clone. The PCR product insertion site is flanked by *EcoR* I sites for convenient identification of insert-containing plasmids. To screen for clones with a specific insert orientation, digest the miniprep DNA with a restriction enzyme with a single cleavage site in the insert DNA and one or a small number of sites in the vector DNA.

Note *Alternatively, positive clones may be identified by PCR analysis of plasmid DNA using the T3/T7 primer pair, or using one primer corresponding to insert sequences and a second primer corresponding to vector MCS sequences.*

Expected Results for the Control Insert Transformation

After plating 10 μ l of the Control Insert transformation reaction, >100 cfu are expected. Greater than 97% of these colonies should be white on agar plates containing X-gal. Plasmid DNA prepared from > 95% of the white colonies should contain the 664-bp Control Insert DNA.

Note *Colonies harboring plasmids containing the Control Insert are white after overnight incubation at 37°C. After prolonged incubation, ~50% of the insert-containing colonies appear light blue. In one orientation, the Control Insert contains an in-frame ATG and ribosome binding site, which allows the synthesis of a LacZ' fusion protein that produces a light blue phenotype after prolonged incubation.*

The presence of the Control Insert is easily verified by digestion of miniprep DNA with *EcoR* I restriction enzyme. DNA fragments expected from *EcoR* I-digestion of plasmids containing the Control Insert are 4252 bp, 557 bp, and 125 bp. Plasmids lacking insert DNA are expected to produce a single detectable *EcoR* I fragment of 4.3 kb.

PREPARATION OF MEDIA AND REAGENTS

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H₂O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave, Pour into petri dishes (~25 ml/100-mm plate)

LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H₂O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave

LB–Ampicillin Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

LB–Kanamycin Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 2.5 ml of 20-mg/ml filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)

2% X-Gal (per 10 ml)

0.2 g of 5-bromo-4-chloro-3-indolyl- β -Dgalactopyranoside (X-Gal)
10 ml of dimethylformamide (DMF)
Store at -20°C
Spread 40 μ l per LB-agar plate

Homework question:

From the number of white colony obtained from each agar plate, calculate the transformation efficiency as (a) per μ g of DNA (b) per plasmid, and (c) per bacterium. Assume the competent bacterial O.D. was 0.6 and 1 O.D. = 10^8 cells/ml; 1 bp = 600 M.W; DNA insert is 500 bp.

Week 4

Plasmid DNA Isolation, Purification and Quantification

Objective

The objective of this weeks' lab is to isolate plasmid DNAs from an overnight bacterial culture containing plasmids with inserted NBS sequence that you cloned last week.

Introduction

Isolating plasmid DNA from *E. coli* is a common routine in research laboratories. There are two major methods for plasmid DNA isolations, boiling prep vs. alkaline lysis. For today's lab, you will use commercially available "mini-prep" kit to perform the widely-practiced plasmid DNA isolation procedure that involves alkaline lysis of bacterial cells. The prep yields fairly clean DNA quickly and ready for DNA sequencing.

Prepare Lysate

1. Centrifuge 1.5ml of bacterial culture for 30 seconds at maximum speed in a microcentrifuge. Discard the supernatant.
2. Add an additional 1.5ml of bacterial culture to the same tube and repeat Step 1.
3. Add 600µl of TE buffer or water to the cell pellet, and resuspend completely.
4. Add 100µl of Cell Lysis Buffer (Blue), and mix by inverting the tube 6 times.

NaOH and SDS (a detergent): The alkaline mixture ruptures the cells, and the detergent breaks apart the lipid membrane and solubilizes cellular proteins. NaOH also denatures the DNA into single strands.

5. Add 350µl of cold (4–8°C) Neutralization Solution, and mix thoroughly by inverting.
Acetic acid and potassium acetate: The acetic acid neutralizes the pH, allowing the plasmid DNA strands to re-anneal. However, the *E. coli* chromosomal DNA, a partially re-annealed tangle at this step, is trapped in the precipitate. The plasmid DNA remains in solution. The potassium acetate also precipitates the SDS from solution, along with the cellular debris.
6. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.
7. Place a PureYield™ Minicolumn into a Collection Tube.
8. Transfer the supernatant (~900µl) to a PureYield™ Minicolumn without disturbing the cell debris pellet and centrifuge at maximum speed in a microcentrifuge for 15 seconds.
9. Discard the flowthrough, and place the minicolumn into the same Collection Tube.

Wash

10. Add 200µl of Endotoxin Removal Wash (ERB) to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 15 seconds.
11. Add 400µl of Column Wash Solution (CWC) to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30 seconds.

Elute

12. Transfer the minicolumn to a clean 1.5ml microcentrifuge tube, then add 30µl of Elution Buffer or nuclease-free water directly to the minicolumn matrix. Let stand for 1 minute at room temperature.
13. Centrifuge for 15 seconds to elute the plasmid DNA. Cap the microcentrifuge tube, quantify, and store eluted plasmid DNA at –20°C for DNA sequencing later.