I: Isolation of Plasmid DNA

II: Cloning of PCR product in a Vector (Plasmid)

*Students will be given general background on DNA isolation techniques to get ready for Plasmid DNA isolation for PCR amplification of a environmental stress related gene NUD7. The amplified DNA will subsequently be cloned in a plasmid vector using a cloning technique called TA Cloning. Subsequently, the Cloned DNA will be introduced in plant cells to investigate the cellular consequences of NUD7 gene expression in plant cells.*

**GENERAL INTRODUCTION TO DNA ISOLATION**

The plasmid DNA (from bacteria) isolation procedures usually make use of the fact that while plasmid DNA is very large relative to most other cellular components, it is much smaller than chromosomal DNA. Thus, conditions which cause shearing and aggregation of chromosomal DNA leave plasmid DNA relatively unharmed. The plasmid DNA can thus be freed from chromosomal DNA by selective precipitation of aggregated chromosomal DNA. The plasmid DNA is then purified from the remainder of the cell constituents remaining in the chromosomal DNA supernatant by precipitation with alcohol. Purification of viral DNA is not covered here, but in general one first purifies the virus, and then the DNA from the virus.

There are numerous protocols used to isolate plasmid DNA. Each of the techniques has advantages and disadvantages. In each of the techniques, it is assumed that you are starting with an overnight culture containing the plasmid of interest.

**PLASMID DNA PREPARATION USING QIAGEN COLUMNS**

**INTRODUCTION:**

Qiagen columns provide an easy, quick way to purify DNA. DNA purified this way is very pure and works well for mammalian transfections. The two major points that must be stressed for these columns to work effectively: 1) do not overload the column by using too much lysed culture and 2) to make sure the columns are rinsed well two times before eluting the DNA. Excess culture volumes contain sufficient protein to interfere with DNA binding. Thus, though initially counter intuitive, one often gets more DNA of higher quality from less culture volume.

PROCEDURE (Taken from the Qiagen Manual)

This protocol is designed for purification of up to 20 μg of high-copy plasmid DNA from

1–5 ml overnight cultures of E. coli in LB (Luria-Bertani) medium. Note: All protocol steps should be carried out at room temperature. Pellet 1.5 ml of overnight grown bacteria by centrifuging 30 sec in full speed (~13,000 RPM)

1. Resuspend pelleted bacterial cells in 250 μl Buffer P1 and transfer to a microcentrifugen tube. Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.
	* Solution P1 contains Tris, and EDTA. Tris is a buffering agent used to maintain a constant pH ( = 8.0). EDTA protects the DNA from degradative enzymes (called DNAses); EDTA binds divalent cations that are necessary for DNAse activity.
2. Add 250 μl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.
	* Solution P2 contains NaOH and SDS (a detergent). The alkaline mixtures ruptures the cells, and the detergent breaks apart the lipid membrane and solubilizes cellular proteins. NaOH also denatures the DNA into single strands.
3. Add 350 μl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. The solution should become cloudy.
	* Solution P3 contains a mixture of acetic acid and potassium acetate. The acetic acid neutralizes the pH, allowing the DNA strands to renature. The potassium acetate also precipitates the SDS from solution, along with the cellular debris. The chromosomal DNA, a partially renatured tangle at this step, is also trapped in the precipitate. The plasmid DNA remains in solution.
4. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.
5. Apply the supernatants from step 4 to the QIAprep spin column by decanting or

Pipetting.

 6. Centrifuge for 30–60 s. Discard the flow-through.

 7. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for

30–60 s.

 8. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.

Important: Residual wash buffer will not be completely removed unless the

flow-through is discarded before this additional centrifugation. Residual ethanol

from Buffer PE may inhibit subsequent enzymatic reactions.

 9. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA,

add 50 μl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

Better than 90% recovery rates (DNA isolation) can be obtained consistently with this protocol

**PCR Amplification of the *Arabidopsis NUD7*Gene**

 **Introduction:**

In the following experiment each student will amplify a full length NUD7 gene using gene specific primers as discussed in the lecture

 **Experiment**

1. You will be carrying out PCR with the isolated plasmid DNA as the template for the amplification reaction.

A) NUD7 plasmid (100 ng) purified by you. Dilute your DNA to 50 ng/l concentration (Dilution will be shown by TA)

1. In the provided PCR tubes add the followings (in order). Note that in any PCR experiment it’s a good idea to add the template last to minimize the opening of tubes containing template that might lead to contamination of other tubes with foreign template DNA.

 Per 50 μl Reaction

 15 l sterile water

 25 μl PCR mix (with buffer, dNTP, Taq polymerase, and salt included)

 4 l NUD7 forward primer (40 pmol)

 4 l NUD7 reverse primer (40 pmol)

 2 l template DNA (Plasmid DNA ~100 ng)

When you have finished assembling these mixes let the TAs know, and they will transfer your samples to the PCR machine and begin cycling with the following program for 35 cycles:

 94oC for 1 min

 55oC for 30 sec

 72oC for 1.5 min (Usually 1 minute for 1 kb DNA amplification)

Then 720C for 10 minutes to complete the extension. At 40C overnight.

4) Pour a 1% agarose minigel (1 gm of agarose in 100 ml of 1X TAE buffer in a clean 500 ml flask (smaller size flask will make the agarose bubble over when boiled). Put the flask in the microwave oven and heat for 1 minute; after one minute, bring out the flask and swirl it around to mix the bubbling agarose (make sure that no bubbling over takes place) and put again in the oven for one more minute. Swirl again after the second minute and then put back in the oven heat for upto 30 more seconds; this will allow the agarose to dissolve completely in the buffer. Cool down until you can touch/hold the flask with bare hand. Pour the warm dissolved agarose on a gel cast and wait until the gel solidifies. Load the PCR products on the gel well. Use 10 l from each reaction + 2 l of 6X loading dye and include one lane of 5 μl of size markers at 50 ng/l (provided) plus 5 μl of TE. If time allows try to run the gel at 50 volts until the orange marker is about at the bottom of the gel. This will take about 2 1/2 hours. You can run the gel at 100 volts but the resolution will probably be poorer.

**Setting Up the TOPO® Cloning Reaction (Adapted from Invitrogen)**

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the

stock salt solution provided in the kit). **Note that the amount of salt added to the**

**TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells.**

• If you are transforming chemically competent *E. coli*, use the stock Salt Solution

as supplied and set up the TOPO® Cloning reaction as directed below.

• If you are transforming electrocompetent *E. coli*, the amount of salt in the

TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM MgCl2 to

prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold

with water to prepare a 300 mM NaCl, 15 mM MgCl2 Dilute Salt Solution. Use

the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed

below.

**Performing the TOPO® Cloning Reaction**

Use the procedure below to perform the TOPO® Cloning reaction. Set up the

TOPO® Cloning reaction depending on whether you plan to transform chemically

competent *E. coli* or electrocompetent *E. coli.*

We will use chemically competent E. coli cells:

**PCR product 2 μl**

**Salt Solution 1 μl**

**Sterile Water add to a final volume of 5 μl**

**TOPO® vector 1 μl**

**Final volume 6 μl**

\*Store all reagents at –20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

**Note:** For most applications, 5 minutes will yield a sufficient number of colonies for

analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be

varied from 30 seconds to 30 minutes. For routine subcloning of PCR products,

30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO®

Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® Competent**

***E. coli***.

**Note:** You may store the TOPO® Cloning reaction at –20°C overnight.

**Transforming One Shot® Competent *E. coli:***

Once you have performed the TOPO® Cloning reaction, you will transform your pENTR™ TOPO® construct into competent *E. coli*. One Shot® TOP10 are included with the kit to facilitate transformation. Protocols to transform chemically competent *E. coli* are provided in this section.

**Preparing for Transformation**

For each transformation, you will need one vial of One Shot® competent cells and

two selective plates.

• Equilibrate a water bath to 42°C (for chemical transformation)

• Warm the vial of S.O.C. Medium from Box 2 to room temperature.

• Warm selective plates at 37°C for 30 minutes.

• Thaw **on ice** one vial of One Shot® cells from Box 2 for each transformation.

**One Shot® Chemical Transformation Protocol**

Use the following protocol to transform One Shot® TOP10 chemically competent *E. coli*.

1. Add 2 μl of the TOPO® Cloning reaction from **Performing the TOPO®**

**Cloning Reaction** into a vial of One Shot® Chemically

Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.

2. Incubate on ice for 5 to 30 minutes.

**Note:** Longer incubations on ice seem to have a minimal effect on transformation

efficiency. The length of the incubation is at the user’s discretion.

3. Heat-shock the cells for 30 seconds at 42°C without shaking.

4. Immediately transfer the tubes to ice.

5. Add 250 μl of room temperature S.O.C. Medium.

6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for

1 hour.

7. Spread 50-200 μl from each transformation on a prewarmed selective plate

and incubate overnight at 37°C. We recommend that you plate two different

volumes to ensure that at least one plate will have well-spaced colonies.