COMPLEMENTATION OF A DWARF ARABIDOPSIS MUTANT DEFECTIVE IN GIBBERELLIN BIOSYNTHESIS

This experiment was first designed by Prof. John Ohlrogge and co-workers (Molina et al., 2008) and it is based on an idea by Prof. Jan Zeewart.

Objective

In this experiment you will complement the phenotype of a dwarf Arabidopsis mutant by cloning the corresponding gene and transferring it back by *Agrobacterium* based plant transformation.

Introduction

During the second half of the 20th century there was a major increase in crop yields, what has been called "Green Revolution". This was due to a combination of improved crop varieties and the use of modern agricultural practices. One of the main improvements in rice and wheat was based on the generation of dwarf or semi-dwarf varieties, which devoted less photosynthate to the stalk and more to the grain and are less prone to "fall over". Breeders selected for this traits from particular lines or plant populations (Hedden, 2003; Khush, 2001)

Only recently the molecular base of this dwarf phenotypes has been discovered. For both wheat and rice the key genes are involved in the biosynthesis or perception of the plant hormone gibberellic acid (GA; Monna et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002).

Gibberellins (there are many different types) are not only involved in stem elongation, but also in flowering, leaf and fruit senescence and, as we will see in the next lab section, in the degradation of storage starch during seed germination. Mutants defective in these phenotypes helped the identification of the genes involved in the biosynthesis and sensing of GAs.

Once a mutation linked to a particular phenotype has been identified it is necessary to confirm that the gene that harbours that mutation is indeed responsible for the phenotype. This experiment is called "complementation" and involves the transformation of the mutant line with a wild type copy of the candidate target gene.

GAs are synthesized via the terpenoid pathway which is used not only for the synthesis of other plant hormones (cytokinins, brassinosteroids, abscisic acid) but also for the production of chlorophyll and carotenoids. Then a large number of different enzymes are involved in the generation of the multiple types of GAs, one of these enzymes is GIBBERELLIN 20-OXIDASE 1 (GA20OX1)

Xu et al. (1995) found that the semi-dwarf mutant GA5 has a mutation in the *GA20OX1*gene which strongly suggests that this mutation might be the cause of the dwarf phenotype. In this experiment you will complement the Arabidopsis mutant *g5-1* (Koornneef, M.,Van Der Veen, J. H. (1980) Internationales Zetischrift fuer Theoretische und Angewandte Genetik :58 (257)) by cloning the gene GA20ox-1 and introducing it back to the mutant via Agrobacterium mediated plant transformation. This experiment involves the following steps:

1. Cloning the target gene from a wild type plant into a binary vector.
2. Transforming the *g5-1* with this "wild type" copy using *Agrobacterium tumefaciens*.
3. Selection of transformed lines and testing for phenotype complementation.

Because it takes some time to generate the transgenic plants we will change the logical order of this experiment and carry out the plant transformation first using a vector that already contains *GA20OX1* that we had previously cloned

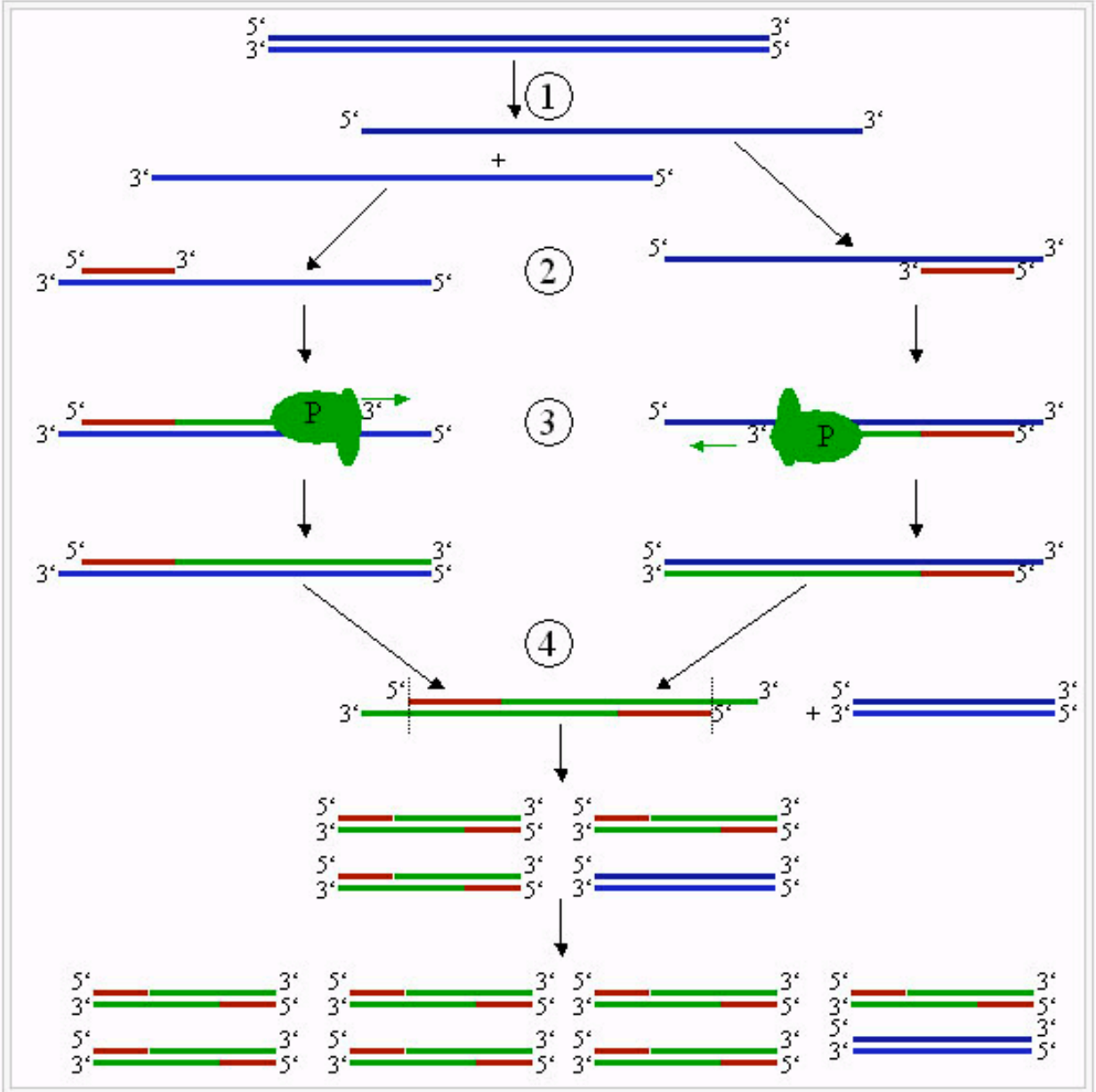

Figure 2: Schematic drawing of the PCR cycle. (1) Denaturing at 94-96°C. (2) Annealing at (eg) 68°C. (3) Elongation at 72°C (P=Polymerase). (4) The first cycle is complete. The two resulting DNA strands make up the template DNA for the next cycle, thus doubling the amount of DNA duplicated for each new cycle.

Figure 1. PCR "refresher" (http://serc.carleton.edu/images/microbelife/research_methods/polymerase_chain_reaction.v2.jpg:)
The red lines are called "primers" . Primers are sequence specific, i.e. they can base pair, to a specific region on the DNA to be amplified. However, primers do not need to match 100%, which is useful when you want to

change the sequence of the gene you are amplifying. We will make use of this property to insert restriction sites to the sides of our gene.

CLONING THE GENE GI20OX1 (AT4g25420).

We will clone the protein encoding section of the GI20OX1 gene into a special DNA vector called binary vector. Binary vectors are circular DNA molecules (plasmids) that are used in the Agrobacterium mediated plant transformation (see lecture on plant transformation for more information or your plant physiology book). We will clone both the CDS using cDNA and the genomic fragment of *GI20OX1* using genomic DNA.

Question 1: what is the difference between CDS and the genomic fragment of a gene?

The cloning involves four main steps:

1. Extraction of DNA and/or RNA from wild type Arabidopsis plants (students will only extract DNA, RNA and the corresponding cDNA will be provided by the TAs).

2. Amplification of the *GI20OX1* gene by PCR using both the genomic DNA as well as the cDNA template. During this process specific restriction sites will be added to the ends of this DNA.

Question 2: will these two PCR products be of the same size?

3. Cutting the binary vector (pMDC32) using the same restriction sites added to the sides of *GI20OX1*.
4. Ligating the DNA of *GI20OX1* with the cut binary vector.

DNA extraction protocol
This is a simple DNA extraction protocol that can be used for downstream PCR applications. For other uses such as restriction digestion a more pure DNA is needed.

Materials to be prepared by TA

- 1.5 ml tubes
- Plastic pestle
- Centrifuge/pipettes
- Isopropanol
- DNA Extraction Buffer:

	Final Concentration	Stock	For 150 ml
○	200 mM Tris HCL (pH 8)	1 M	?
○	250 mM NaCl	2.5 M	?
○	25 mM EDTA	0.5 M	?
○	0.5% SDS	10 %	?
○	H2O	100%	?

Question 3: What is the volume of the different stock solutions necessary to make 150 ml of DNA extraction buffer?

Part I
1. Add 300 µl of DNA extraction buffer to a 1.5 ml tube.
2. Cut a small piece of leaf (0.5 cm^2) and transfer it to the tube.
3. Homogenize tissue in the extraction buffer with plastic pestle.
4. Centrifuge tube 15 min at maximum speed (~13000 rpm).
5. Transfer the supernatant to a new 1.5 ml tube.

6. Add 1 volume of 100% isopropanol, mix well and place overnight at -20C (or 30 min on ice)

Part II

7. Spin tube for 20 min at maximum speed (~13000 rpm).
8. Remove the supernatant, and leave the tube open to dry for 10 min. Make sure to remove all the isopropanol.
9. Add 50 μl of double distilled water, vortex well and transfer to a 37C water bath for 10 min. You can keep the DNA overnight at 4C if not needed immediately.
10. Centrifuge sample for 5 min at maximum speed and transfer the supernatant to a new tube. Use DNA for your PCR reaction or store it at -20C.

Cloning:

We will insert the *GI20OX1* CDS and the *GI20OX1* genomic fragment into the binary vector pMDC32 using the restriction enzymes AscI/PacI in front of the *35S* promoter (see the map of the vector, find the restriction sites and the *35S* promoter). For a nice explanation on how restriction enzymes work go to http://www.dnalc.org/resources/animations/restriction.html

Note on the 35S promoter *(http://www.cambia.org/daisy/promoters/242/g1/250.html):*
At the beginning of the 1980s, Chua and collaborators at the Rockefeller University isolated the promoter responsible for the transcription of the whole genome of a Cauliflower mosaic virus (CaMV) infecting turnips. The promoter was named CaMV 35S promoter ("35S promoter") because the coefficient of sedimentation of the viral transcript whose expression is naturally driven by this promoter is 35S. It is one of the most widely used, general-purpose constitutive promoters.
The 35S promoter is a very strong constitutive promoter, causing high levels of gene expression in dicot plants. However, it is less effective in monocots, especially in cereals. The differences in behavior are probably due to differences in quality and/or quantity of regulatory factors.

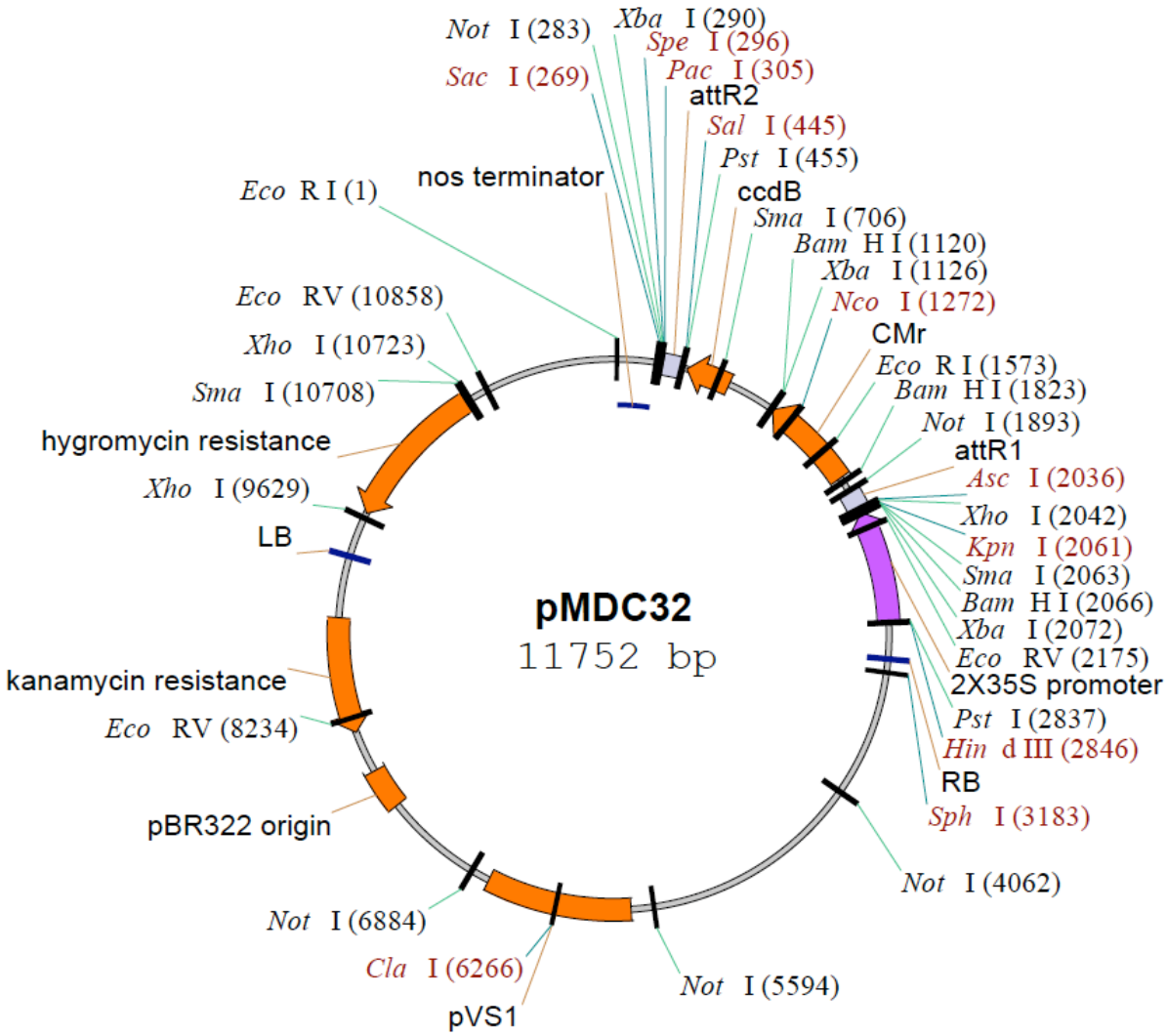

Figure 1. Restriction map of the pMDC32 binary vector. Enzymes are in italics. In red are enzymes that cut this vector only once. LB: Left border of the T-DNA; RB: Right border of the T-DNA. pBR322, origin of replication in E. coli. pVS1, Origin of replication in *Agrobacterium*. AttR2/AttR1/ ccdB/CMr, other sequences we are not interested in right now.

Materials to be prepared by TA

- Sterile double distilled water (100 ml for all groups)
- Primers: AscIGI5F and GI5RPacI (TA see below for concentration, give each group an aliquot)
- For PCR: dNTPs, primers, Pfu Turbo polymerase, (Stratagene) PCR tubes, sterile tips, TA give each group an appropriate aliquot).
- PCR machine (borrow from Farre lab).
- Agarose gel running set up and power-supply, 1 per group.
- Restriction enzymes: AscI (Order from NEB) and PacI (Biochemstore), Buffer 4 (NEB), BSA (NEB): -20C enzyme box.
- T4 DNA Ligase + buffer (Promega, get from Biochemstore), TA give each group an appropriate aliquot.
- Liquid LB medium: see Plant transformation protocol
- LB+ kn plates: see Plant transformation protocol
- Tubes for bacteria growth: get from Biochemstore
- Miniprep kit: Promega (Biochemstore)
- Gel extraction kit: Promega (Biochemstore)
- Competent E. coli cells: DH5alpha, (Invitrogen, Biochemstore) **TA: they are in Farre lab (-80C, Eva's shelf). Give each group 2x 50 µl aliquots.**
- Cell spreaders (1 per group)(get sterile from Farre lab)
- Bunsen burner (1 per group), or use sterile bench if Bunsen burners are not available.
- Spray bottle with ddH20, to clean quartz cuvette.
- Quarz cuvettes (Room 255, in the cabinet behind the photometer)
- SYBR DNA staining
- Digital camera to take pictures of gels (use Schemske lab set up).
- DNA loading buffer (-20C PLB316 box), TA give each group an appropriate aliquot.
- 1 Kb+ DNA ladder (-20C PLB316 box) , TA give each group an appropriate aliquot.
- Heating block or waterbath.
- cDNA (from Farre lab, -20C PLB316 box; give each group one 2 ul aliquot in PCR tube)
- genomic DNA (for back up in case there is a problem with the extraction, -20C PLB316 box)
- ~13 ml culture tubes for bacteria (sterlize glass tubes or get disposable from Biochemstore), each student will need 1.
- pMDC32 vector (give each group one 1 µl aliquot, ~300 ng, in 1.5 ml microfuge tube). http://botserv1.uzh.ch/home/grossnik/curtisvector/index_2.html. Curtis and Grossniklaus (2003).
- TAE buffer: Tris-acetate EDTA buffer:
  1x = 40 mM Tris-acetate, 1mM EDTA
  for 1L 10x: 48.4g Tris base; 11.42 ml glacial acetic acid, 20 ml 0.5 M EDTA
  You might need 4 L for 6 groups.

Note: For all procedures that include enzymes always mix the buffer and water first, and add enzymes last if possible, proteins might denature if they are not in the appropriate solution.

1. Set up the PCR reactions: Each group will set up 2 reactions, one with cDNA as substrate and one with genomic DNA as substrate. Set up the reactions on ice (i.e. keep all solutions on ice).

Final Concentration	**Stock Conc.**	**Volume for 50 µl**
1x Turbo Pfu buffer	Buffer (10x)	
75 µM dNTPs	dNTP (1.25 mM each)	
125 nM Primer 1	Primer 1 (2.5 µM)	
125 nM Primer 2	Primer 2 (2.5 µM)	
0.05 U/µl Turbo Pfu Polymerase	Pfu (0.833U/µl)*	
2µl/50 µl cDNA or DNA	cDNA or DNA	
sterile ddH_20	sterile ddH_20	

***TA: for Pfu you need to dilute the original stock for 2.5 U/µl 1:3 in 1x PCR buffer to achieve 0.833 U/µl, do this just before use and keep mix on ice, don't freeze this diluted enzyme. Make sure the primers and the dNTP stocks have the right concentration.**

Question 4: What is the volume of the different stock solutions necessary to set up a 50 µl PCR reaction?
Question 5: What are dNTPs?

2. Run the PCR reaction using a PCR machine (TA freeze PCRs at -20C until the next day).

3. Purify PCR product (to remove dNTPs and polymerase) using the Promega kit (see Appendix for protocol).
4. Cut the PCR product (use whole PCR product) and binary vector (300 ng) using DNA restriction enzymes.

Set up digestion reactions two restriction digestion reactions:

Final Concentration	**Stock Conc.**	**Volume for 50 µl (PCR product)**	**Volume for 50 µl (Vector)**
PCR product	(use all)	39 µl	
or 300 ng vector	300 ng/µl		1 µl
1x NEB Buffer 4	10x Buffer 4		
1x BSA	25x BSA*		
0.2 U/µl AscI	5 U/µl*		
0.2 U/µl PacI	5 U/µl*		
ddH_20		add ddH_20 to make 50 µl	add ddH_20 to make 50 µl

*** TA:**
a) Dilute 100x BSA stock to 25x BSA stock with ddH20, you can prepare this in advance and freeze it if you want.
b) Dilute enzymes 1:2, to achieve 5 U/µl (original stock is 10 U/µl) in 1x Buffer 4, prepare just before use and keep mix on ice, don't freeze the diluted enzyme.

Question 6: What is the volume of the different stock solutions necessary to set up a 50 µl restriction digestion to cut the PCR product?

Question 7: What is the volume of the different stock solutions necessary to set up a 50 µl restriction digestion to cut the vector?

b) Cut vector and PCR product for 1-2h at 37C:

5. While digestion is going on, prepare a 1% (w/v) agarose gel with TAE buffer (50 ml total volume) including SYBR DNA staining. Each group will run 1 gel.
6. Run restriction in the gel, run also one lane with 10 µl DNA ladder for size control.
7. Cut out bands PCR product and cut vector from the gel and elute DNA from the gel piece using a gel purification kit (Promega).

Question 8: How many bands (i.e DNA fragments) do you expect to see for the digestion of your PCR product?
Question 9: How many bands (i.e DNA fragments) do you expect to see for the digestion of your vector? See pMDC32 restriction map.

8. Now you will set up a ligase reaction in which the PCR product will be ligated to the cut vector to form a new circular plasmid.

Final Concentration	**Stock Conc.**	**Volume for 20 µl**
1x Ligase Buffer	10x	2 µl
400 U Ligase	400U/µl	2 µl
Cut Insert (=PCR product)*		
Cut Vector*		
ddH_20		add ddH_20 to make 20 µl

* Add as much insert and vector as possible, maintaining a molecular ratio of 3:1 respectively: You need 3 molecules of insert per 1 molecule of vector. You need to estimate these amounts from the bands to saw in the gel. The intensity of the bands in the gel correlates with the amount of DNA present. Remember that the vector is ~10x bigger than your insert; i.e. 1 molecule of vector will stain as much as 10 molecules of your PCR product.
*Set up also a control reaction with in which you omit the insert (but keep exactly the same amount of vector). This control will give you an indication of how much uncut vector you have in your reaction.

10. Incubate overnight at 16C.

11. Transform 5 µl of ligase reaction into competent E. coli cells (DH5alpha, Invitrogen):

1. Thaw a 50 µl aliquot of competent cells on ice.
2. Add 5 µl of ligase reaction and mix by holding the top of the tube and tapping the bottom (**Do not mix by pipetting up and down**, competent cells are very sensitive).
3. Incubate the vial on ice for 30 min.
4. Heat-shock for exactly 20 seconds in the 37°C water bath. Do not mix or shake.
5. Place vial on ice for 2 min.
6. Add 500 µl of LB liquid medium.
7. Tape vials horizontally to a shaking incubator and incubate 1 h at 37C at 225 rpm.

TA: Please also Transform also 0.5 µl of pMDC32+genomic *GI20OX1* and 0.5 µl of pMDC32+CDS *GI20OX1* (see -20C PLB316 box); for this you will only need to use 25 µl of cells per transformation and plate only 15 µl of the culture after the 1 h incubation. In case the student reactions don't work, you will set up miniprep cultures from the colonies resulting from these transformations.

12. Plate cells on selection plates using a sterile spreader (LB+kanamycin [50 μg/ml]). Once the plate has dried, invert it and incubate overnight at 37C.

TA: take out plates from the incubator the next day.

13. TA: Set up overnight miniprep cultures (3ml) in LB +kn medium (use sterile toothpicks or tips). Grow the cultures in shaker at ~225 rpm at 37C. Set up 2 mini cultures per group, each from one colony. Keep the plates at 4C and show them to the students next day.

14. Count how many colonies you got in each plate.

Question 10: Under which circumstances would you get colonies in the control ligation reaction (the one without insert)? List all the options you can think about.

15. Isolate plasmid DNA from the overnight bacteria cultures using the miniprep kit (Promega). See instructions at the end of this file.

Question 11: What is the difference between the plasmid DNA and the bacterial genomic DNA that allows their separation in the plasmid preparation protocol? Look it up if you do not know!

Now we need to check whether the ligation was successful and the PCR product was introduced in the vector. We do this by cutting the isolated plasmid and testing whether it will give the restriction pattern that we are expecting.

15. Measure DNA concentration using a photometer and quartz cuvettes. We do this using the Beer-Lambert law (see GLOSSARY in the syllabus for an explanation of this law) and the specific absorbance of DNA which is 1 OD = 50 μg DNA/ml.

Add 4 μl of plasmid to 400 μl of ddH_20. In between samples rinse cuvette using ddH_20 in a spray bottle. Use ddH_20 as blank.

a. Set up photometer to 260 nm
b. Zero photometer using blank
c. Measure absorbance of sample
d. Calculate DNA concentration using the specific absorbance of DNA

Question 12: What is the concentration of DNA if the absorbance measured as described above was 0.3? Use the specific absorbance of DNA to do this calculation [an absorbance of 1 (i.e. 1 OD) corresponds to a concentration of 50 μg/ml].

16. Check plasmid for the right insertion by restriction digestion: Each group will do two.

a. Set up reactions

Final Concentration	**Stock Conc.**	**Volume for 50 μl**
300 ng DNA/reaction	what is your plasmid conc.?	calculate
1x NEB Buffer 4	10x Buffer 4	
1x BSA	25x BSA*	
0.2 U/μl AscI	5 U/μl*	
0.2 U/μl PacI	5 U/μl*	
ddH_20		add ddH_20 to make 50 μl

*** TA:**
a) Dilute 100x BSA stock to 25x BSA stock with ddH20, you can prepare this in advance and freeze it if you want.
b) Dilute enzymes 1:2, to achieve 5 U/µl (original stock is 10 U/µl) in 1x Buffer 4, prepare just before use and keep mix on ice, don't freeze the diluted enzyme.

Question 13: What is the volume of the different stock solutions necessary to set up a 50 µl restriction digestion?

b) Transfer restriction digestion reactions to 37C, and incubate for 1 h.

17. Run restriction digestions in a 1% (w/v) agarose gel. Use SYBR DNA staining. Don't forget to add the DNA ladder for size control.

Question 14: How many bands and of what size do you expect to see in the gel if your ligation was successful?

18. If you get the bands you were expecting, we will send some of that plasmid DNA for sequencing.

DNA sequencing

We will send our samples for sequencing to the MSU Research Technology Support Facility (RTSF; (http://rtsf.msu.edu/custom-sequencing). The method they will be using is based on the "Sanger Method". There is a nice explanation of how this works at:
http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/Obenrader/sanger_method_page.htm

I expect you to understand this method. I you don't, come and ask.

We will sequence the *GI20OX1* gene from both sides, i.e. we will set up two reactions one with a forward primer and one with a reverse primer. For each sequencing reaction mix 1µg of plasmid DNA and 30 pmol primer in a total volume of 12µl. We will use ddH_20 to achieve the final volume.

Question 15: What would be the reaction mix if you had a plasmid concentration of 0.3 ng/µl and a primer stock with a concentration of 25 µM; i.e. what volume of your plasmid solution, what volume of your primer solution and how much ddH_20 do you need to mix together?

NOTE: Once we have our final plasmid we can transform *Agrobacterium* cells. We will not do this in class, but for your information you do it the same way as you transform *E. coli* cells. Due to time constrains you have already performed the plant transformation using *Agrobacterium* cells harboring the GI20OX1 gene. However, you should know the order of how things actually work.

PLANT TRANSFORMATION

Note: you performed this experiment with Dr. Weise.

Dr. Weise
410 Biochemistry
email: weisesea@msu.edu

Introduction:

During the second half of this course you will be working on an ongoing project to genetically engineer a plant! As you will learn in the course genetic engineering is not something that is quick (at least not yet) but rather the result of methodical careful work. Because of the time involved (mainly the time it takes for plants to grow) we will have to get started with part of this project a little early so that your plants will be ready when you begin the second part of the course with Dr. Farre. In this lab you will "transform" mutant *Arabidopsis* plants that have their gene coding for a gibberellin oxidase enzyme "messed up" by the addition of a T-DNA insert in this gene that contains nonsense or junk DNA. The DNA that you will transform your plants with contains a good copy of the gibberellin oxidase and will "rescue" the mutant plants.

You will learn more about gibberellins and gibberellin oxidase later in the course. As a short introduction gibberellins or gibberellic acid (GA) are a class of plant hormones or signalling substances that regulate growth and development. One of the primary functions of gibberellin is to stimulate internode stem elongation. Plants that are deficient in GA are usually shorter in height. The enzyme gibberellin oxidase was important in the Green Revolution of the late forties and fifties when modern agricultural research and breeding practices were brought to bear in confronting food shortages faced by South America and Asia. The result of the Green Revolution was higher yielding plants and the introduction of more modern agronomic practices. These higher yields were often achieved by breeding for strains of rice, corn, and wheat that were shorter in stature thus less prone to lodging and had more flowering (grain yielding) stalks per plant. Although they did not know it at the time many of the shorter higher yielding cultivars that were breed for during the green revolution were the result of changes to various genes in the GA synthesis pathway one of them being your new favourite gene gibberellin oxidase.

It is not trivial to introduce DNA into higher organisms and have it stably incorporated into the host organism's chromosomes. There are multiple techniques for doing this, but the method of choice for generating most transgenic plants is the use of a common soil bacteria *Agrobacterium tumefaciens* or Agro for short. *Agrobacterum* is normally a pathogenic bacterium to plants and causes crown gall disease in thousands of dicotyledonous plant species. *Agrobacterium* is able to mediate the only known natural example of DNA transport between kingdoms: it transforms plants cells with a DNA fragment called the T-DNA (transferred DNA), which is part of the large tumor inducing (Ti) plasmid. The T-DNA is transferred to the plant nucleus where it is stably integrated into the plant genome (Fig 1). The Ti plasmid encodes also for the virulence (vir) genes, which are necessary for the transfer of the T-DNA to the host plant cell. The expression of these genes is induced by acetosyringone, a substance produced by wounded plant cells. The T-DNA that is transferred to the plant genome encodes for enzymes that synthesize plant growth hormones (auxins and cytokinins), which lead to uncontrolled growth and tumor formation. The T-DNA also encodes for enzymes that generate various amino compounds called opines which the bacteria use as their main source of carbon and nitrogen. Neither the plant or other soil microorganism can metabolize the opines and thus its production creates a biological niche for the *Agrobacterium*.

Now that you understand how *Agrobacterium* works in out the cold cruel "real world" how are we going to use it to transform our Arabidopsis plants with the gibberellin oxidase gene. For starters we are going to use an *Agrobacterium* that has been disarmed. When we say disarmed what we mean is that we have removed the genes in the Ti plasmid that are harmful to the plant. These include the genes that encode for the auxin and cytokinin producing enzymes and the genes that encode for the enzymes that synthesize opines. In place of these genes we will put the gene or genes that we want to incorporate into the plants genome. In our case it is the gene for gibberelin oxidase. We also have to include a selectable marker gene. We are going to transform the flowers of

plants that are just about to produce seed. What we hope will happen is that the *Agrobacterium* will insert our gene of interest into the female gametophyte that is developing in the pistol and the resulting seed will have our gene inserted into it's genome (Fig 1). That is what we "hope" will happen. *Agrobacterium* is not 100% efficient. That is, it will not insert DNA into every female gametophyte. Furthermore if you look carefully, the plants you are going to transform already have some siliques (seed pods) on them. These seeds have already developed and therefore will not be transformed. So we need to select only the seeds that been transformed by the Agro. To do this we will put a gene in our disarmed Agro that encodes for antibiotic resistance. This way we can harvest all the seeds from our transformed plants and place the seeds on Petri dishes which contain agar infused with antibiotic. The seeds that survive are the seeds that have been transformed and contain the antibiotic resistance as well as our gibberelin oxidase gene. Given all the things that the Ti plasmid has to do: vir genes, gibberelin oxidase gene, antibiotic resistance gene our plasmid is getting pretty big. As you will find out later in the course the bigger the plasmid the harder it is to get your genes into it. So to make the plasmid smaller to facilitate getting your gene into it we will split the plasmid into two. The first being the Ti plasmid which will contain your gene of interest and antibiotic resistance and the second often called the helper plasmid will contain the vir genes and a gene for antibiotic resistance.

Okay we have our gibberelin oxidase gene and we have our *Agrobacterium* transformation system now what about our hapless victim, the plant we are going to transform? We are going to use *Arabidopsis thaliana*. *Arabidopsis* is a weed in the Brassicaceae family that is native to Europe and central Asia. In the last 20 years *Arabidopsis* has become what we refer to as a "model" organism. That is, it is heavily studied by thousands of lab across the world with the hope that by focusing our effort on understanding one plant as much as possible we can apply the combined knowledge gained to other species. *Arabidopsis* has a very small genome for a plant ≈ 27,000 gene and is diploid. Because of its small genome *Arabidopsis* was the first plant to have it's genome sequenced. *Arabidopsis* is small in size as well so it is easy to grow lots of plants, often needed for statistical significance. Finally *Arabidopsis* is easy to grow. If you have not worked with *Arabidopsis* yet chances are very likely that at some point in your botanical career you will.

Protocol:

You will work in groups of 2 but each person is responsible for having all the data in his or her lab notebook. You will use mutant *Arabidopsis* plants that have had their gibberelin oxidase gene "knocked-out." Normally these plants would grow extremely slowly and might not ever flower. We have rescued this slow growing phenotype by spraying the plants with exogenous GA. Look at the WT plants that have not had their GA synthesis knocked-out and compare those to the mutant plants that have been sprayed with GA and the mutant plants that have not been sprayed with GA. Take a picture of these plants to document the phenotype and record your observations in your lab notebook. We will take the mutant plants and dip them into a solution containing disarmed Agro with our gibberelin oxidase gene. Wear gloves and try not to slop *Agrobacterium* on yourself. Not only does it not smell nice, but it contains the antibiotics kanamycin and tetracycline.

The protocol we are using is adapted from Steve Clough and Andrew Bent, University of Illinois at Urbana-Champaign (Clough and Bent, 1998). This protocol is extremely simple and work well as long as you have healthy plants. With this method you should be able to achieve transformation rates above 1% (one transformant for every 100 seeds harvested from *Agrobacterium*-treated plants).

The following steps were carried out by your TAs and instructor prior to lab but are included here so you have a complete understanding of the protocol and can write about it in your lab report.

A. Mutant and WT Plant Growth

Materials

- RediEarth
- Growth chamber
- 10 mM GA_4 stock in 90% ethanol, use this to make 0.1 mM stock in 5% ethanol

Procedure

6-7weeks before needed sew wild-type Arabidopsis (ecotype Landsberg erecta, Ler) and *ga5-1* mutants (missing the gibberelin oxidase enzyme) in pots with "RediEarth" (brand of potting soil) pre-treated with nutrient water. Cover pots with a nylon window screen mesh to hold soil during floral dipping procedure. Stratify seeds in a cold room for 4 days at 4°C to ensure uniform germination. Plants are grown under white fluorescent light (80-100 μmol m^{-2} s^{-1}) in a 16 hr light / 8 hr dark photoperiod at 20-22°C.

Starting at 4-weeks after sewing and continuing weekly, spray *ga5-1* mutants with an aqueous solution of 0.1 mM **GA_4**, containing 5% (v/v) ethanol. Do not spray one or two of the pots with mutant plants as controls.

B. Floral Dip of Arabidopsis

Materials

- Make 1 L LB (Luria broth) medium by adding 25 g LB Broth (Difco, Luria-Bertani) to 1 L H_2O autoclave. When LB has cooled to around 50°C add 1 ml of 50 mg/ml kanamycin (final concentration 50 μg/ml). Add 1 ml of 5 mg/ml Tetracycline (stock is prepared in 70% ethanol) (final concentration 5 μg/ml). **Note Tetracycline is light sensitive.** Transfer 50 ml to 250 ml flasks and autoclave.
- Seven 250 ml flasks autoclaved
- Glycerol stock *Agrobacterium* carrying a plasmid coding for the gi20ox1 (AT4g25420)
- Empty blue tip boxes, 1 per group to mix infiltration solution
- Infiltration medium (prepare fresh): for one transformation: 150 ml dH_2O add 7.5 g sucrose (5%), + 30 μl silwet L-77 (200 μl/L).

Procedure

1. Streak agrobacterium on LB+kanamycin (50μg/ml) plate from glycerol stock at least 3 days before needed. Note: if you are setting up cultures handle all bacteria always under a flame/Bunsen burner or sterile hood.
2. Three days prior to plant transformation, inoculate a 2 mL liquid culture of *Agrobacterium* carrying a suitable binary vector and incubate at 28 °C with vigorous agitation. Use LB medium containing antibiotics that select for both the Ti and the T-DNA plasmids.
3. After 2 days inoculate 50 mL LB medium (6 flasks) with 1 mL of the preculture and incubate again with vigorous agitation for a further 24 hours at 28 °C.
4. Stop watering the plants and allow the soil to dry out a little, so that it will be less prone to falling out of the pots during dipping.

**** Okay students This is the part your doing in this lab ****

5. Pellet *Agrobacterium* by centrifuging at 2000g (6000 rpm in a GSA rotor, or equivalent) for 10 minutes. If possible, centrifuge at room temperature. Note that the cell pellet is pink.
6. Resuspend the cell pellet in 50 mL of LB medium. Mix with 150 mL infiltration medium.
7. Transfer the *Agrobacterium* suspension to a convenient vessel for dipping plants, e.g., a box of 1 ml disposable pipette tips or a 400-mL beaker.
8. Invert a pot of plants and dip the inflorescence flowers into the suspension. Rest the pot on the edge of the beaker and allow the plants to soak for ~30 seconds. The same suspension can be used for ten or more pots if enough volume was prepared. Try to avoid contamination of the soil with *Agrobacterium*, which produces a rather unpleasant smell.
9. After dipping, wrap pots with saran wrap for the next 24 hours.

**** Okay tune back out your TA and instructor with take care of the following steps ****

10. After 24 hours, remove the cover, and water them.
11. For higher rates of transformation, plants may be dipped two or three times at seven-day intervals. We suggest one dip two days after dipping, and a second dip one week later. Do not dip less than 6 days apart.
12. After about 3-4 weeks, collect seeds.

SELECTION OF TRANSFORMED SEEDLINGS

The plant transformation protocol does not work with 100% efficiency, which means that we have to be able to select the transgenic seedlings from a pool of untransformed ones. Remember that the same happened when you transformed E. coli cells with the plasmid. Similarly to selection in bacteria we can select for transformed seedlings by growing them on antibiotic. Within the left and right border primers of the T-DNA in the pMDC32 vector there is a hygromycin resistance gene (see pMDC32 map). This gene will be expressed in the transgenic plants and lead to resistance to hygromycin. Plants are sensitive to many antibiotics because chloroplasts are from bacterial origin.

We will first select transformed seedlings by growing them in sterile Murashige & Skoog media containing hygromycin. *gi5-1* mutants display no obvious phenotype at the seedling stage and therefore we need to transfer the transgenic seedlings to soil and let then grow for a couple of weeks. As you saw and documented previously *gi5-1* mutant plants are very late flowering. By the end of the course you will be able to see whether the complementation experiment worked.

Materials (to be prepared by TA):

- 50% bleach + 0.1% triton X-100 (50 ml, for all groups)
- Sterile water: give 50 ml to each group
- MS (Murashige-Skoog) + Kanamycin [50μg/ml] solid media in large petridishes (give each group 3 plates, 1 L makes ~10 plates)
- 4x 1.5 ml microfuge tubes with ~ 100 μl of T1 seeds (first generation transformants).
- Sterile 1ml tips.
- Optional: Waterbath

MS+ kanamycin selection plates: **TA prepare 2 L, Jan has large petridishes in a storage room.**

Component	*Amount to add to make 1 L*
1x MS salts	4.33 g
(Bacto) Agar	7 g
ddH_2O	to make 1 L

*Combine MS salts and water, and stir to dissolve. Adjust pH to 5.7 with KOH. Add agar to a 1 L bottle and add the MS solution to it. Mix well, but try not to touch the bottle lid with solution. Sterilize by autoclaving.
*Turn sterile bench on and clean surface with 70% ethanol 10 min before needed.
*When cooled enough you can hold the bottle in your hand add the antibiotics (50 μg/ml final concentration, previously filter-sterilized solution) in the sterile bench. You can place the media in a 60C waterbath for cooling. You can Mix well but avoid getting air bubbles in the medium. Pour plates in the sterile hood (Keep bag where plates came in). When medium has solidified, place plates upside down in the bag and store at 4C.

Note: "Murashige and Skoog medium [...] is a plant growth medium used in the laboratories for cultivation of plant cell culture. [MS] was invented by plant scientists Toshio Murashige and Folke K. Skoog during Murashige's search for a new plant growth regulator. It is the most commonly used medium in plant tissue culture experiments" (http://en.wikipedia.org/wiki/Murashige_and_Skoog_medium).

Procedure:
We first need to sterilize the seeds. The surface of seeds contains a lot of fungal spores, which tend to grow faster than your plants in MS medium. Seeds have a very chemically resistant coat that allows us to treat them with harsh chemicals that kill bacteria and fungi on the surface but do not damage the seeds. Due to their seed coat and low water content seeds are in general very resistant, for example you can freeze *Arabidopsis* seeds in liquid nitrogen (−196 °C; −321 °F) for 10 min and they will survive!
Then, you will plate the sterile seeds using sterile flow hood on MS plates containing hygromycin.

1. Sterilize *Arabidopsis* seeds:
* Turn on the sterile flow hood.
* Wash seeds for 10 min in 50% bleach with 0.001-0.2% SDS or Triton X-100 (at .1 %). For 100 ul of seeds use a 1.5 microcentrifuge tube and 1 ml of bleach solution.
* Rinse seeds five times with 1 ml sterile water to ensure that all of the bleach has been removed. Do this in the sterile flow hood.
* Resuspend seeds in 500 μl sterile water.

2. Plate seeds on selection plates:
Use a sterile pipette to transfer seeds to MS plates. Try to spread out the seeds as much as possible.

3**. Transfer seedlings to soil**.
After two weeks, transfer transgenic seedlings to small pots containing RediEarth using forceps. Transgenic seedlings will be bigger than the unstransformed ones and will have started growing primary leaves. Cover the pots with a plastic hood for 2 days. We need to do this because plants grown in tissue culture, i.e. under high humidity, have a reduced cuticula (the waxy coat on the surface of plants) and they wilt rapidly if left uncovered. By covering them we give them some time to build their cuticula.

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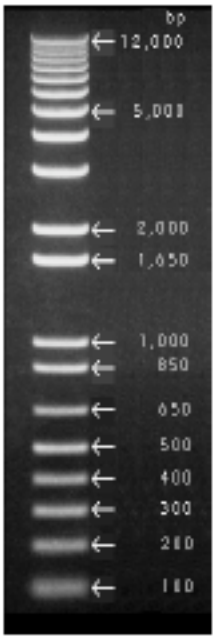
Additional commentary can be found by searching the Arabidopsis newsgroup archives: http://genome-www.stanford.edu/cgi-bin/biosci_arabidopsis

APPENDIX

Invitrogen 1Kb+ ladder



1 Kb Plus DNA Ladder
0.7 µg/lane
0.9% agarose gel
stained with ethidium bromide

Structure of Fragments in 1-Kb Increments:

5'-GATCC——— G
G ——— CCTAG-5'
(996 bp)$_n$

Notes:
During 1% agarose gel electrophoresis with Tris-acetate (pH 7.5) as the running buffer, bromophenol blue migrates together with the 500 bp band.

The 1650 bp band is generated from pUC. The bands smaller than 1000 bp are derived from lambda DNA.

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Wizard Plus Minipreps DNA Purification System:

PureYield™ Plasmid Miniprep System

INSTRUCTIONS FOR USE OF PRODUCTS A1220, A1221, A1222 AND A1223

Quick PROTOCOL

Solution Preparation

Before lysing cells and purifying DNA, prepare the Column Wash Solution by adding ethanol. Cap tightly after addition. See Technical Bulletin #TB374 for detailed instructions.

DNA Purification by Centrifugation

Prepare Lysate

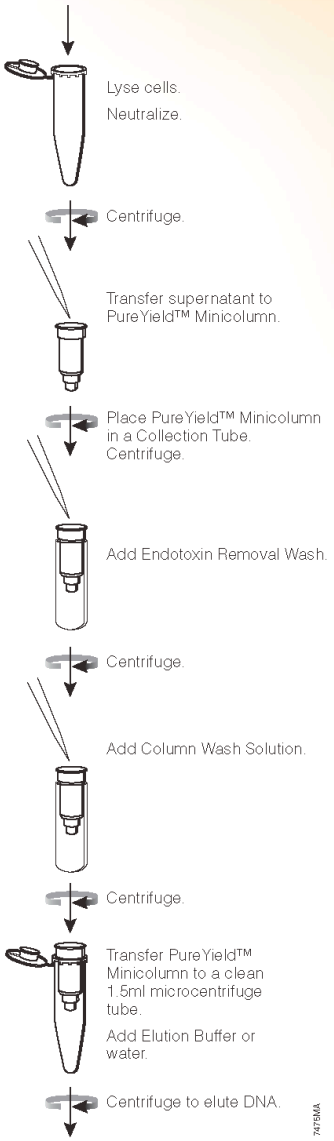
1. Add 600µl of bacterial culture to a 1.5ml microcentrifuge tube.
   Note: For higher yields and purity use the alternative protocol below to harvest and process up to 3ml of bacterial culture.
2. Add 100µl of Cell Lysis Buffer (Blue), and mix by inverting the tube 6 times.
3. Add 350µl of cold (4–8°C) Neutralization Solution, and mix thoroughly by inverting.
4. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.
5. Transfer the supernatant (~900µl) to a PureYield™ Minicolumn without disturbing the cell debris pellet.
6. Place the minicolumn into a Collection Tube, and centrifuge at maximum speed in a microcentrifuge for 15 seconds.
7. Discard the flowthrough, and place the minicolumn into the same Collection Tube.

Wash

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

Elute

10. 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.
11. Centrifuge for 15 seconds to elute the plasmid DNA. Cap the microcentrifuge tube, and store eluted plasmid DNA at –20°C.



Alternative Protocol for Larger Culture Volumes

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. Proceed to Step 2 of the standard protocol above.

For complete protocol information see Technical Bulletin #TB374, available at: **www.promega.com/tbs**

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INSTRUCTIONS FOR USE OF PRODUCTS A1220, A1221, A1222 AND A1223

Quick PROTOCOL

DNA Purification by Vacuum

Prepare Lysate

1. Transfer 1.5ml of culture to a 1.5ml microcentrifuge tube
   Note: If you wish to process larger volumes of bacterial culture (up to 3ml) use the alternative protocol provided below.
2. Centrifuge at maximum speed in a microcentrifuge for 1 minute.
3. Remove and discard medium.
4. Resuspend the cell pellet in 600µl of TE buffer or water.
5. Add 100µl of Cell Lysis Buffer (Blue), and mix by inverting the tube 6 times.
6. Add 350µl of cold (4–8°C) Neutralization Solution, and mix thoroughly by inverting.
7. Centrifuge at maximum speed in a microcentrifuge for 3 minutes. Place a PureYield™ minicolumn on a Luer-Lok® adapter of a VacMan® or VacMan® Jr Laboratory Vacuum manifold
8. Transfer the supernatant (~900µl) into a PureYield™ Minicolumn.
9. Apply vacuum pulling the lysate through the column.

Wash

10. Add 200µl of Endotoxin Removal Wash (ERB) to the minicolumn. Allow the vacuum to pull the solution through the column.
11. Add 400µl of Column Wash Solution (CWC) to the minicolumn. Allow the vacuum to pull the solution through the column. Release the vacuum, and remove the PureYield™ Minicolumn.

Elute

12. Place the column in a 2ml collection tube, and centrifuge at maximum speed in a microcentrifuge for 1 minute.
13. Transfer the minicolumn into 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.
14. Centrifuge for 15 seconds to elute the plasmid DNA. Cap the microcentrifuge tube, and store eluted plasmid DNA at –20°C.

For complete protocol information see Technical Bulletin #TB374, available at: **www.promega.com/tbs**

Alternative Protocol for Larger Culture Volumes

1. Centrifuge 1.5ml of bacterial culture for 30 seconds at maximum speed in a microcentrifuge.
2. Discard the supernatant.
3. Add an additional 1.5ml of bacterial culture to the same tube. Repeat Steps 1 and 2.
4. Add 600µl of TE buffer or water to the cell pellet, and resuspend completely.
5. Proceed to Step 5 of the standard protocol above.

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DNA Purification by Centrifugation

Gel Slice and PCR Product Preparation

A. Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10µl Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C until gel slice is completely dissolved.

B. Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA

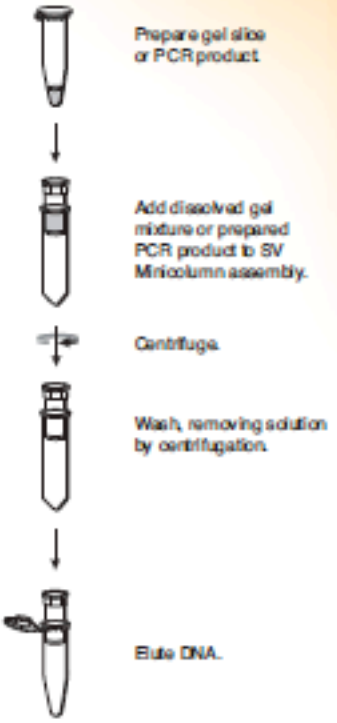
1. Insert SV Minicolumn into Collection Tube.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Washing

4. Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × *g* for 5 minutes.
6. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × *g* for 1 minute.
9. Discard Minicolumn and store DNA at 4°C or –20°C.



Additional protocol information is available in Technical Bulletin #TB308, available online at: **www.promega.com**

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