Lab 2: *ARABIDOPSIS THALIANA* DNA

Lab 2.1: DNA EXTRACTION

Goal for this lab:

Extract DNA from *Arabidopsis thaliana* leaves.

Introduction

In this lab, we will extract DNA from our model organism, *Arabidopsis thaliana.* We will be using the DNA we make today throughout the rest of the semester, so this is an important activity!

In any DNA purification (or extraction) protocol, there are three important considerations:

1. We must break up the tissues and cells to release the DNA.
2. We must prevent the DNA from being degraded by cellular enzymes during the purification.
3. We must get rid of cellular components that are not desired—in this case, proteins, lipids, carbohydrates (cell walls), and RNA.

Break up tissues and cells

We will cause tissue and cell disruption in three ways. The first is mechanical grinding. We will use a ball mill to grind frozen leaf tissue into a fine powder. Freezing (in liquid nitrogen) makes the leaf tissue brittle and easy to grind. This breaks up the extracellular matrix in which the cells reside and allows better access of our chemicals to the cells themselves. The second method of disruption is chemical. The DNA extraction buffer that we use contains a high concentration of a detergent, sodium dodecyl sulfate (SDS). You will recall that detergents have both hydrophobic and hydrophilic moieties, and thus will bind both lipid and water. This detergent will solubilize the lipid bilayers in the cells, so that the plasma membrane and the internal membranes are dissolved. In addition, we use the metal chelating compound ethylene diamine tetra-acetic acid (EDTA), which tightly binds calcium (Ca^{++}) and magnesium (Mg^{++}) effectively locking it away from cellular components. In the absence of Ca^{++} and Mg^{++}, proteins denature, and membranes lose their integrity. The last disruption is high temperature. This encourages the detergent and EDTA to work efficiently and hastens cellular disintegration. This high temperature step is critical for efficient cellular disruption and release of DNA.

Prevent DNA degradation

We prevent DNA degradation by inhibiting the enzymes (deoxyribonucleases, or ‘DNases’) that degrade DNA. Conveniently, DNAses require Mg^{++} as a co-factor, so, by chelating the Mg^{++} with EDTA, we inhibit the activity of DNases. Thus, it is imperative to get the EDTA containing DNA extraction buffer well mixed with the ground tissue as soon as possible.

Get rid of undesirable molecules

We will use two methods to accomplish this. Both rely on differential solubility of specific macromolecules in particular solutions. Proteins and complex carbohydrates (such as those found in cell walls) are insoluble in solutions containing high concentrations of potassium acetate (KOAc). By adding KOAc, we force the proteins and carbohydrates to precipitate (become solid and fall out of solution) while the nucleic acids will remain in solution. Nucleic acids are insoluble in solutions containing high levels of sodium plus the alcohols ethanol (EtOH) or isopropanol (ISOP), while lipids are quite soluble in these conditions. Proteins and carbohydrates are somewhat soluble in alcohol. By adding alcohols to a salty solution, we can force the nucleic acids to precipitate, while leaving the lipids, and the remaining carbohydrates and proteins, in solution. The main problem with alcohol precipitation of nucleic acids is that the sodium precipitates along with the nucleic acids. This is undesirable, and so we always rinse nucleic acid pellets with 70% EtOH (containing no salts) to remove the sodium from the pellets prior to dissolving the nucleic acids in the next solvent.

Materials

Reagent	Concentration			pH	also known as
DEB	100	mM	NaCl	8.0	Sodium chloride
(DNA Extraction Buffer)	50	mM	Tris	8.0	Trishydroxymethylaminomethane
	25	mM	EDTA		Ethylene diamine tetra-acetid acid
	1	%	SDS		Sodium dodecyl sulfate
	10	mM	β-ME		β-mercaptoethanol
$T_{10}E_5$	10	mM	Tris	8.0	
	5	mM	EDTA		
$T_{10}E_1$	10	mM	Tris	8.0	
	1	mM	EDTA		
EtOH	95	%			Ethanol
EtOH	70	%			Ethanol
ISOP	100	%			Isopropanol
KOAc	5	M			Potassium acetate (CH3COOK)
NaOAc	3	M		5.2	Sodium acetate (CH3COONa)
LN_2					Liquid Nitrogen

1.5 ml tubes

Paper and tape

Miracloth and funnel

Pot of arabidopsis

Tissue grinder

Method

Before you begin, read through the directions, and get everything out that you will need so you don't have to fumble for it in the middle of some critical step in the extraction procedure. Each student will do one extraction (i.e., two extractions per pair).

SAFETY: *LN_2 is very very cold, –320ºF (–196ºC), and will severely damage tissue that it contacts! Use extreme caution while handling to avoid freezing injuries. (I usually wear double gloves…)*

1. Label two 1.5mL microfuge tubes for your two Col-0 (wild-type) samples.
2. Add 2 metal beads to each labeled 1.5mL tube and pre-cool the tubes by placing in liquid nitrogen (LN_2). **Make sure the tubes are closed tightly before throwing into LN_2 or LN_2 will leak in and the tubes will pop next time you take them out!**
3. Cut 2 leaves per plant (~100-200mg) and immediately put them into the 1.5mL tubes. Do not overload the tubes! Close the tubes and keep them in LN_2. Do not let the tissue thaw once it is frozen.
4. Quickly, without letting the tissue thaw, move the 1.5mL tubes from LN_2 into prechilled blocks for the tissue grinder. Be sure that the blocks are balanced with an equal number of tubes in each.
5. Shake the blocks in the grinder for 1 minute. Check carefully to ensure that grinding occurred as expected. Immediately place tubes back in LN_2.
6. Remove one tube at a time, into a rack. Add 600µl DEB and mix vigorously by vortexing until the powder has mixed with the liquid. Mix again before heat treatment.
7. Incubate the tubes at 65ºC in a water bath for 15 minutes.
8. Place the tubes on ICE and add 250µl 5M KOAc. Mix well by inverting the tubes repeatedly. Incubate ON ICE for 20 minutes.
9. Centrifuge at maximum speed for 10 minutes 4ºC. Remove your tubes from the centrifuge and notice the pellet consisting of tissue debris and precipitated protein. REMEMBER, you are trying to GET RID of the pellet in this step!
10. Carefully take up the supernatant (the liquid on top of the pellet) without disturbing the pellet. Transfer it through a Miracloth filter in a funnel into a fresh tube containing 0.6 mL of 100% isopropanol. Mix well by inverting the tube gently but repeatedly. Precipitating nucleic acids *may* be clearly visible at this point.
11. Centrifuge at maximum speed for 5 minutes 4ºC. Remove your tube from the centrifuge. A pellet should be readily visible at this point. REMEMBER, this time, YOU WANT YOUR PELLET—the pellet is the nucleic acids— including your DNA. Remove supernatant by tipping over the tube, being sure the pellet remains at the bottom.
12. Rinse your pellet carefully with 200µl 70% EtOH. (If pellet has moved, you can spin the samples for 1 minute at 5000 rpm).
13. Remove as much liquid as humanly possible (a second centrifuge spin of the "empty" tube can be useful here -- if needed, do this at 1000 rpm for 30 seconds, then pipet out any remaining EtOH).
14. Let the pellet air dry for a few minutes. Getting rid of the EtOH is essential before proceeding to the next step. Leave the tube open to dry for at least 2 minutes.

15. Add 100µl of $T_{10}E_5$ and leave the tubes on ice until pellet is redissolved (~10-15 min). We still use a high concentration of EDTA at this step, because not all the proteins are gone yet, so nucleases could still attack our DNA. Once completely redissolved transfer the liquid into a new labeled 1.5 mL microtube.
    Failure to redissolve is more often a sign of carbohydrate in the prep (which will never dissolve…) than of poor dissolving of DNA. Happily, carbohydrate typically does not interfere with typical downstream analyses like PCR or RE digestion.
16. Add 10µl of 3M NaOAc and mix gently by pipetting. Add 200µl of 100% EtOH and mix gently by inverting the tube until phases are completely mixed. Fluffy whitish clouds of DNA should be visible.
17. Centrifuge at maximum speed for 2 minutes. Alternatively, you can use a 1mL pipette tip like a spatula to lift out the nucleic acid fluff and transfer it into a clean, dry, labeled 1.5mL microtube. If you centrifuged, remove supernatant by tipping over the tube, being sure the pellet remains at the bottom.
18. Wash the pellet with 200µl of 70% EtOH. Centrifuge at 1000 rpm for 30 seconds, then remove as much EtOH as possible. Again, you can alternatively use a pipette tip to transfer the nucleic acid fluff into a clean dry labeled 1.5mL microtube.
19. Let the tube air dry for a few minutes (~15 min). DO NOT OVERDRY! *A good way to tell is when the edges of the pellet are turning clear, but the center is still opaque.*
20. Resuspend the pellet in 50µl of $T_{10}E_1$. We use a low concentration of EDTA now because our nucleic acid preparation is fairly pure—protein contamination is minimal now. However, we keep some EDTA around 'just in case'. If we had too much EDTA in our final solution, we could never do subsequent enzymatic manipulation of our DNA.
21. Label your tube carefully with your group initials, the date, and "Col-0 gDNA". Put it in a rack. We will leave the tubes in the fridge to redissolve overnight.
22. Prepare a freezer box with your group name on it in label tape. We will store the samples once completely redissolved to -20ºC.

Write in your lab notebook what you did, either "exactly as in manual", or "as in manual, except that …". Give the date, and what label you put on the tube. Later on in the semester, when you will need this DNA again, all this will help you remember (or figure out) what's in the tube you just put in the freezer.

Clean-up

Alcohols are safe to go down the drain only when they are less than 10%. Run plenty of water while discarding alcohols so that this dilution will be achieved.

Save your reagents. If you have to make DNA again later, you'll be glad to have them. You will use your $T_{10}E_1$ again.

Put the tubes that contained any β-mercaptoethanol in the marked bucket in the hood.

Put plant debris and used potting soil in the bucket labeled "Compost".

Save the pot the plants were grown in.