DNA EXTRACTION PROTOCOL

1. Dry roots at 37° C for 3 days.
2. Store dried roots in a 2 ml cryotube at -80°C until DNA extraction.
3. Crush the dried and frozen roots by beadbeating (Mini-Beadbeater, Biospec Products) with sterile glass beads for 30 seconds or until a fine powder formed.
4. Immediately place samples on ice.
5. Add 1.5 ml of 2x CTAB buffer (2% CTAB, 1% PVP, 0.1 M Tris pH 8.0, 1.4 M NaCl, 0.02 M EDTA) to the cryotube.
6. Place in water bath at 65°C and invert to mix every 20 minutes for 1 hour
7. Add 600 µl of $CHCl_3$:iso-amyl (24 chloroform:1 iso-amyl alcohol) and vortex for ~30s.
8. Centrifuge for 10 minutes at max speed (13,000 g).
9. Pull off the top layer (~700 µl) and place in a new labeled tube. Discard the middle and lower layers.
10. Add 0.5X volume 95% EtOH (ethanol) (for 700 µl supernatant, add 350 µl). Gently mix/invert for 1 minute.
11. Clean extracted DNA using a Qiagen DNeasy tissue kit (Qiagen Inc., Valencia, CA, USA) using the following protocol:
    a. Add sample to the Qiagen spin column on the vacuum pig.
    b. Add 500 µl of Buffer AW1 and allow to vacuum through.
    c. Add 500 µl of Buffer AW2 and allow to vacuum through.
    d. Place the Qiagen spin column into collection tube and centrifuge at max speed for 1 minute. Toss the collection tube and place the Qiagen filter into a 1.5 ml tube.
    e. Add 50 µl of Buffer AE to the Qiagen spin column. Let the samples sit for 1-2 minutes.
    f. Centrifuge samples for 1 minute at max speed. The DNA is now in the 1.5 ml tube.
    g. Store in -20°C freezer.

TIP – Make sure your roots are colonized first! Be aware of potential PCR inhibitors in the extraction. The PVP is used to lessen the influence of inhibitors.

PCR PROTOCOL – *Marker:* 18S rDNA, *Primer set*: AM1/NS31

1. Dilute DNA extracts 1:10 or 1:100 in sterile double distilled water.
2. PCR reactions are each 20 µl consisting of 12.4 µl of dH2O, 0.2 µl of 2.5 U Pfu Turbo DNA polymerase (to create blunt-ended fragment), 2 µl of manufacture's buffer (Stratagene), 2 µl of 10x dNTPs, and 0.2 µl of each 50µM primer, and 3 µl of DNA extract.
3. PCR conditions are as follows (same as described by Helgason 2002):

Thermocycler conditons:		
Step 1	95° C	1 min
Step 2	58° C	1 min
Step 3	72° C	2 min
Step 4	Go to 1	9 times
Step 5	95° C	30 sec
Step 6	58° C	1 min
Step 7	72° C	3 min
Step 8	Go to 5	19 times
Step 9	95° C	30 sec
Step 10	58° C	1 min
Step 11	72° C	10 min
Step 12	10° C	Forever

TIP – The dilution of DNA extract also helps to limit problems with inhibitors. Increase the volume of the PCR rxn to increase amount of product. Do gradient PCR to optimize annealing temps.

BLUNT-ENDED CLONING PROTOCOL

1. In order to improve cloning efficiency, PCR products (approx. 550 bp) are first gel purified according to the manufactures instructions (QIAquick Gel Extraction Kit, Qiagen) and eluted in 50 μl (MAX) of nuclease free water.
2. Concentrate the eluted purified products by drying in a SpeedVac (Savant) and then resuspend products in 10 μl of nuclease free water.
3. Purified and concentrated PCR products are then cloned into pPCR-Script Amp SK(+) and transformed into Escherichia coli XL10-Gold Kan Ultracompetent cells (Stratagene) using the following procedure:

Ligation reaction

Prepare the 65°C water bath

Add the following components *in order* in a 0.5 ml microcentrifuge tube to carry out a **1/2 reaction**:

0.5 μl	pPCR-Script Amp SK(+) vector (10 ng/μl)
0.5 μl	PCR-Script 10X reaction buffer
0.25 μl	of 10 mM rATP
2.75 μl	your PCR product **or** 2.75μl ctrl PCR
0.5 μl	Srf I restriction enzyme (5U/μl)
0.5 μl	T4 DNA ligase (4U/μl)
5μl	Final volume

Then:

- Mix the ligation reaction gently and incubate for 1 hour at RT
- Heat the ligation reaction for 10 minutes at 65°C
- Store the ligation reaction on ice until ready for transformation

Transformation reaction

Prepare ahead of time:

- Thaw competent cells on ice
- Chill 14-ml BD Falcon polypropylene round-bottom tubes
- Preheat SOC in a 42°C water bath. Keep water bath on for heat shocking

Begin transformation

1. Gently mix cells by hand. Aliquot 25 μl of the cells into a chilled Falcon tube for each of your reactions*.
2. Add 1.0ul β-mercaptoethanol to cells and swirl contents gently
3. Incubate cells on ice for 10 minutes, swirling gently every 2 minutes
4. Add 2ul of your ligated product to the transformation reaction and swirl gently (plasmid control dilute 1:10 first)
5. Incubate reaction on ice for 30 minutes
6. Heat pulse the tubes in 42°C water bath for 30 seconds. *It is critical that the time and the temperature are exact*
7. Incubate tubes on ice for 2 minutes
8. Add 250 μl preheated SOC medium to each tube and incubate tubes at 37 °C for one hour with shaking at 225-250 rpm

Plating

- Pipette 75 μl or less (or more) of the transformed cells and spread onto the LB-carb, X-gal, IPTG plates.
- If you are running controls, plate the following amounts:
  - Plate 10 μl of the PCR test insert + 100 μl media onto LB-carb, X-gal, IPTG plates

* If you are running controls, prepare Falcon tubes and cells for the following reactions: (1) your experimental ligation reaction (2) ligation reaction containing the PCR test insert and (3) the pUC18 control plasmid

BLUNT-ENDED CLONING PROTOCOL (CONT.)

    - Plate 5 μl of the pUC18 control reaction + 100 μl media onto LB-carb, X-gal, IPTG plate
- Incubate plates overnight at 37°C. For blue-white colony screening, incubate for 17 hours
- Choose white colonies for examination. White colonies may turn blue after a few days.

Picking Clones
- Pick white colonies with sterile tooth pick and "waggle" into PCR master mix

Saving Clones
1. Add 150μl of LB broth with 100 μg/ml of Carbenicillin to each well of cell culture plate
2. Pick white colonies and add toothpick to wells of culture plate (after waggle if PCR at same time) and leave until incubation – cover top with AirPore tape sheets
3. Incubate overnight @ 37°C
4. Add 50 μl of 80% glycerol, mix gently, add metal tape, and put into –80° C freezer

Solutions/Stocks for Cloning Plates

1. Carbenicillin (Ampicillin analogue for longer storage)
    a. Stock = 100 mg/ml dH_2O (filter sterilize solution)
    b. Plate = 0.1 mg/ ml LB-agar media
2. X-gal
    a. Stock = 20 mg/ml in 100% dimethyl formamide (DMF) – LIGHT SENSITIVE and toxic!!
    b. Plate = 40-50 μg/ml LB-agar media
3. IPTG
    a. Stock = 200 mg/ml dH_2O(filter sterilize solution)
    b. Plate = 250-350 μg/ ml LB-agar media

    *STORE STOCKS at –20° C

PCR of Clones

<u>Recipe for PCR reaction</u>	
dH_2O	12.5 μl
10X buffer	2.0 μl
10X dNTPs	2.0 μl
20uM T3 primer	0.2 μl
20uM T7 primer	0.2 μl
2.5U taq polymerase	0.1 μl
DNA template	<u>Waggle</u>
	17 μl

<u>Thermocycler Conditions:</u>		
Step 1	94° C	10 min (lyses cells)
Step 2	95° C	2 min
Step 3	50° C	45 sec
Step 4	72° C	1:30 min
Step 5	95° C	30 sec
Step 6	50° C	30 sec
Step 7	72° C	1:30 min
Step 8	Go to 5	29 times
Step 9	72° C	7 min
Step 10	10° C	forever

***Verify your PCR products on a 1.5 % agarose gel**

TIP – This protocol maximizes the amount of PCR product added by eliminating water, you can adjust this if you have good PCR product concentrations. With this kit insert:vector ratio is very important. If necessary, decrease the amount of vector if your product concentration is low. Running controls is critical when beginning to figure out why things aren't working (or mysteriously stopped working).

CLEANING PCR PRODUCTS - ExoSAP-IT (USB) ¼ **reaction (96-well plate)**

1. Make Exosap-dH_20 cocktail: for each well you want 0.5 μl of Exosap and 1 μl of dH_20 (for 10 reactions, you want a cocktail with 5 μl of Exosap and 10 μl of dH_20)
2. Add 1.5 μl of the cocktail to each well.
3. Add 3.5 μl of PCR product to each well.
4. Vortex plate for ~5 seconds to mix samples.
5. Spin down plate very briefly.
6. Add to thermocycler and run the Exosap program:

Thermocycler conditons:		
Step 1	37° C	45 min
Step 2	80° C	15 min
Step 3	10° C	forever

SEQUENCING REACTIONS with BIG DYE v3.1 (Applied Biosystems)

We use sequencing reactions that are 1/8 strength manufacturer's instructions and they work fine. Make sure to do the sequencing reaction in a sequencing plate. You can make up the master mix first and then add the clean PCR product. If your PCR product is weak, you can put in 2 μl and no dH_2O.

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Reagents	1 rxn	50 rxns	100 rxns	Cycler Program
Clean PCR product	1 μl	----	----	1. 96°C, 1 min
Big Dye v3.1 seq. mix	1 μl	50 μl	100 μl	2. 96°C, 10 sec
Big Dye seq. Buffer (5X)	1 μl	50 μl	100 μl	3. 50°C, 5 sec
1.25 μM primer	1 μl	50 μl	100 μl	4. 60°C, 4 min
dH_2O	1 μl	50 μl	100 μl	5. Go to 1 24x
				6. 10°C hold

SEQUENCING REACTION CLEANING (EDTA/EtOH method)

1. Add 1.25 μl 125 mM EDTA to each well.
2. Add 15 μl 100% EtOH to each well.
3. Vortex briefly to mix.
4. Incubate at room temperature for 15 minutes.
5. Spin at 2254 g (max speed) for 35 minutes.
6. Invert plate onto paper towel (well face down), centrifuge for 1 min at 700 g
7. Add 15 μl 70% EtOH to each well.
8. Spin at 2254 g for 10 minutes.
9. Invert plate onto paper towel (well face down), centrifuge for 1 min at 700 g.
10. Allow to dry at room temperature in dark (i.e. covered in foil).

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