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Quick Reference: Supply list

Reagents

- 0.25 N HCl
- 0.25 N NaOH
- 0.25% Nonidet P-40
- 0.5 M Tris pH8
- 10 mM dNTP set
- 50% glycerol
- BP Clonase II kit (Invitrogen # 11789-100)
- DNA polymerase kits (these are similar products):
    - iProof (Bio-rad cat# 172-5302)
    - Hot Start Phusion DNA polymerase+ HF buffer (NEB # F-540L)
- Exonuclease I (GE Healthcare Life Sciences #E70073Z)
- Arabidopsis Columbia gDNA (10 ng/µL)
- Gentamicin (Sigma Cat# G1397)
- Kanamycin
- Lab tape and sharpie
- LB Agar and media
- LR Clonase II kit (Invitrogen #11791-100)
- pDONR207 donor vector, 150 ng/µL
- pYXT2 destination vector, 150 ng/µL
- Rifampicin (10 mg/mL stock)
- Shrimp Alkaline Phosphatase (GE Healthcare Life Sciences #E70092Z)
- Silwet L-77
- SOC (Invitrogen)
- TE pH 8.0
- TOP10 competent E. coli (or equivalent strain without F' episome)
- YET/2 Media

Consumables

- Air pore strip
- Deep well block
- Foil seals
- Four chamber plates (VWR #62407-071)
- Freezer plates
- Heat seals (AB Gene AB-0745)
- Parafilm
- Petri Dish 150X15 mm (Falcon #1058)
- PCR Plates
- Reservoirs, 100 mL
- Sterile toothpicks
- Tips for Multi-channel pipettes:
    - Matrix : 125 µL (Matrix #7441); 1 mL (TIGR # 1060)
    - Rainin: 10 µL (Rainin #SS-L10); 300 µL (Rainin #SS-L300)

Other supplies

- 28C Shaker, 37C Shaker
- Cold block for setting up reactions
- Gel running+visualization supplies (TBE, agarose, etbr, etc)
- Glass beads (Zymo research #S1001)
- Sterile hood
- Multi-channel pipettes

Transgenic Plant Definitions and Numbering Scheme

T0 = refers to the plant being dipped in Agro.
T1 = seeds and plants from the T0 plant
T2 = seeds and plants from the T1 plant

Transgenic plant numbering scheme:

T0:	**Agro Plate#.Well.Attempt #.Transformation Plant #**
T1:	**Agro Plate #.Well.Attempt #.Transformation Plant #.Plant #**

Primers

Universal attB primers

attB1adaptor_promo
5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3'

attB2adaptor_promo
5'- GGGGACCACTTTGTACAAGAAAGCTGGGT-3'

---

pDONR207 sequencing primers

pDONR207Forward
5'- TCGCGTTAACGCTAGCATGGATCTC-3'

pDONR207Reverse
5'- GTAACATCAGAGATTTTGAGACAC-3'

Promoter PCR

Supplies

- gDNA (10 ng/µL)
- Promoter Primer dilutions
- Universal attB primers for promoter cloning
- Milli-Q H2O
- 10 mM dNTP set
- One of following DNA polymerase kits (these are the same products):
    - Hot Start Phusion DNA polymerase+ HF buffer(NEB)
    - iProof (Bio-rad)
- PCR Plates
- Heat seals (AB Gene AB-0745) or foil seals
- Cold block for setting up reactions
- Gel running+visualization supplies (TBE, agarose, etbr, etc)

PCR1

1) Pipette 1 µL of each 2.5 µM promoter primer stock into PCR plate
2) Prepare master mix:

Reagent	**per rxn**		**96 rxns (plus 15%)**	
Milli-Q H2O	13.4	µL	1474	µL
5X HF buffer	4.0	µL	440	µL
10 mM dNTPs	0.40	µL	44	µL
gDNA (10 ng/uL)	2.0	µL	220	µL
Phusion/iProof	0.2	µL	22	µL

Pipette into each sample: 19 µL
Total reaction volume: 20 µL

Cycling conditions:

Program: <MAIN>, BUTD20
CHECK CYCLING PARAMETERS BEFORE STARTING~!

In calculated mode:
1X: 98C 30 sec
1X: 98C 10 sec, 63C 30 sec, 72C 2 min
1X: 98C 10 sec, 62C 30 sec, 72C 2 min
1X: 98C 10 sec, 61C 30 sec, 72C 2 min
1X: 98C 10 sec, 60C 30 sec, 72C 2 min
1X: 98C 10 sec, 59C 30 sec, 72C 2 min
9X: 98C 10 sec, 58C 30 sec, 72C 2 min
1X: 72C 10 min
1X: 4C forever

PCR2

1) Pipette 10 µL PCR1 into new PCR plate. This is template for PCR2.
2) Prepare PCR2 master mix:

Reagent	**per rxn**		**96 rxns (plus 15%)**	
Milli-Q H2O	18.8	µL	2068	µL
5X HF buffer	6	µL	660	µL
10 mM dNTPs	0.8	µL	88	µL

AttB Primer mix10 uM	4.0	µL	440	µL
Phusion/iProof	0.4	µL	44	µL

Pipette into each sample: 30 µL
Total reaction volume: 40 µL

Cycling conditions:

Program: <MAIN>, BUATT20
CHECK CYCLING PARAMETERS BEFORE STARTING~!

1X: 98C 30sec
19X: 98C 10sec; 56C 30 sec; 72C 2 min
1X: 72C 10 min
1X 4C forever

Gel Electrophoresis

1) Pour a large 1.5%, 1X TBE gel for 96 samples
2) Run 10 µL PCR products on gel with ladders at the ends of the rows
3) Take picture of gel and save file on network.
4) Check PCR products. Should have 1 strong band around 2-2.5 kB in most samples.

Storage of PCR products

PCR products are stable in -20C. However, they become less efficient in BP reactions with storage time (after 1-2 weeks in the freezer). Don't store the PCR product long before doing BPs.

BP Entry Cloning

Supplies

- PCR2 attB PCR product
- pDONR207 donor vector, 150 ng/µL
- TE pH 8.0
- BP Clonase II kit (Invitrogen # 11789-100)
- PCR plates
- Heat seals (AB Gene AB-0745) or foil seals
- Cold block for setting up reactions
- Multi-channel pipette and tips

Propagating pDONR207

Supplies: LB Chlor Gent plates, LB Chlor Gent Media, Qiagen Spin Miniprep Kit

We have glycerol stocks of pDONR207. This plasmid is propagated in a special E coli strain that is resistant to the effects of *ccdB* expression. pDONR207 is a high copy plasmid.

Culture strain: Streak culture out on LB Chloramphenicol (30 µg/mL) + Gentamicin (7 µg/mL) plate from glycerol stock. Grow overnight at 37C. Pick a few colonies for culture in 50 mL LB chlor + gent. Prepare DNA using Qiagen mini-preps (use ~10 preps and combine at end) or Qiagen Midi Kit. Need 150 ng/µL plasmid DNA concentration for BP reactions.

BP reactions

1) Dilute 5 uL PCR2 products in 25 uL H2O.
2) Set up BP reactions:

Reagent	**Each rxn**		**96 rxns (plus 20%)**
Diluted PCR product	1.0	µL	---
TE pH 8.0	0.75	µL	86.25 µL
pDONR 207 (150ng/µL)	0.25	µL	28.75 µL
~remove BP Clonase from -80 freezer, put on ice, vortex twice~			
BP Clonase II mix	0.50 µL		57.5 µL

Spin plate down in plate spinner and incubate at 25C overnight in PCR machine (~20hours).

Proteinase K treatment

After 20 hour BP incubation, stop reactions with Proteinase K.
Spin down plate.
Add 0.3 µL proteinase K to each sample. Store proteinase K in deli fridge.
Seal plate and spin down.
Incubate at 37C, 10 min.
Spin plate.

Storage of BP reactions

BP reactions can be stored in freezer after stopping with proteinase K.

E. coli Transformations with BP reactions

Supplies

- TOP10 competent E. coli cells (or equivalent strain that does not have F' episome)
- SOC (Invitrogen)
- LB Gent plates (7 µg/mL Gent)
- Sterile glass beads
- 37C Shaker
- Gentamicin (Sigma Cat# G1397)
- YET/2 Media
- Reservoir, P-300 multi-channel pipette and tips, deep well block, air pore strip, sterile toothpicks, freezer plates, heat seals or foil seals

Transformation reactions

1) Take out enough cells from the -80C freezer to transform each BP reaction with 24 µL cells.
2) Thaw cells on ice.
3) Put BP reactions on ice in cold block.
4) When cells have thawed, pipette 24 µL cells into each BP reaction.
5) Incubate on ice for 20 min.
6) Heat shock cells in PCR machine at 42C, 30 sec.
7) Ice 2 min.
8) Add 175 µL room temp SOC (Invitrogen) to each sample.
9) Incubate at 37C, 225 rpm for 1 hour.
10) Plate out 50 µL on LB Gent with glass beads.
11) Incubate plates upside down at 37C overnight. Take plates out in morning.
12) If any of samples do not have colonies, re-plate those samples with all the remainder of the transformation reaction.
13) Take pictures with epi-light and save images.
14) Store plates in plastic bag or seal with parafilm and store at 4C until colony picking.

Picking colonies

1) Prepare two deep well blocks with 500 µL YET/2 + 7 µg/mL Gent. Write lib_id clearly on 2 sides of deep well block
2) Pick 1 colony into each well. Maintain same well numbering as the primer plate numbering. Skip wells for samples that did not produce any colonies. Keep the toothpick in the well to track which colonies have been picked.
3) Remove toothpicks and cover block with air pore strip.
4) Place blocks in one layer in 37C incubator, no shaking. Do not stack blocks. Incubate overnight.
5) The following morning take out blocks. Check for growth, write down if some samples did not grow.
6) Make glycerol plate copy of blocks to keep at TIGR. Write lib_id on 2 sides of freezer plate. Check orientation of plate.
7) Pipette 30 µL 50% glycerol into freezer plate.
8) Pipette 120 µL culture into freezer plate. Pipette up and down a few times to mix.
9) Freeze deep well blocks and TIGR glycerol copies at -80C.
10) Make primer dilutions of pDONR207 primers.
11) Send deep well blocks for sequencing with pDONR207 primers:
    a. Prepare 250 µL 5 pmol/µL each pDONR207forward and pDONR207reverse primer from primer stocks.

IMPORTANT: request DNA to be sent back to TIGR.

LR Destination Cloning

Supplies

- LR Entry clone DNA from JTC
- pYXT2 destination vector, 150 ng/µL (prepared in-house)
- TE pH 8.0
- LR Clonase II kit (Invitrogen #11791-100)
- PCR plates
- Heat seals (AB Gene AB-0745) or foil seals
- Cold block for setting up reactions
- Multi-channel pipette and tips

Propagating pYXT2

Supplies: LB Chlor Kan plates, LB Chlor Kan Media, Qiagen Spin Miniprep Kit

We have glycerol stocks of pYXT2. This plasmid is propagated in a special E coli strain that is resistant to the effects of *ccdB* expression. pYXT2 is not a high copy plasmid so expect plasmid yield to be lower.

Streak culture out on LB Chlor (30 µg/mL) + Kan (50 µg/mL) plate from glycerol stock. Grow overnight at 37C. Pick a few colonies for culture in 50 mL LB chlor + kan. Prepare DNA using Qiagen mini-preps (use ~10 preps and combine at end) or Qiagen Midi Kit. Need 150 ng/µL plasmid DNA concentration for LR reactions.

LR reactions

1) Select list of entry clones to work with from the database.

2) Thaw entry clone plasmid DNA from JTC on bench.

3) Once DNA is thawed, pipette 1 µL DNA into new PCR plate.

4) Set up LR reactions:

Reagent	**Each rxn**	**96 rxns (plus 20%)**
Entry clone plasmid DNA	1.0 µL	---
TE pH 8.0	0.75 µL	86.25 µL
pYXT2 (150ng/µL)	0.25 µL	28.75 µL
~remove BP Clonase from -80 freezer, put on ice, vortex twice~		
LR Clonase II mix	0.50 µL	57.5 µL

Spin plate down in plate spinner and incubate at 25C overnight in PCR machine (~20hours).

Proteinase K treatment

After 20 hour LR incubation, stop reactions with Proteinase K.
Spin down plate.
Add 0.3 µL proteinase K to each sample. Store proteinase K in deli fridge.
Seal plate and spin down.

Incubate at 37C, 10 min.
Spin plate.

Storage of LR reactions

LR reactions can be stored in freezer after stopping with proteinase K.

E. coli Transformations with LR reactions

Supplies

- TOP10 competent E. coli cells (or equivalent strain that does not have F' episome)
- SOC (Invitrogen)
- LB Kan plates (50 µg/mL)
- Sterile glass beads
- 37C Shaker
- 50% glycerol
- 96 well freezer plates
- Kanamycin
- YET/2 Media
- Reservoir, P-300 multi-channel pipette and tips, deep well block, air pore strip, sterile toothpicks, freezer plates, heat seals or foil seals

Transformation reactions

1) Take out enough cells from the -80 freezer to transform each LR reaction with 24 µL cells.
2) Thaw cells on ice.
3) Put LR reactions on ice in cold block.
4) When cells have thawed, pipette 24 µL cells into each LR reaction.
5) Incubate on ice for 20 min.
6) Heat shock cells in PCR machine at 42C, 30 sec.
7) Ice 2 min.
8) Add 175 µL room temp SOC (Invitrogen) to each sample.
9) Incubate at 37C, 225 rpm for 1 hour.
10) Plate out 50 µL on LB Kan with glass beads.
11) Incubate plates upside down at 37C overnight. Take plates out in morning.
12) If any of samples do not have colonies, re-plate those samples with all the remainder of the transformation reaction.
13) Take pictures with epi-light and save images.
14) Store plates in plastic bag or seal with parafilm and store at 4C until colony picking and mating with Agrobacterium.

Picking colonies

1) Read "Tri-parental mating with Agrobacterium" section of protocol. Pick colonies when there is time for Agro mating.
2) Prepare two deep well blocks with 250 µL LB media + 50 µg/mL Kan. Write lib_id clearly on 2 sides of deep well block
3) Pick 1 colony into each well. Maintain same well numbering as the primer plate numbering. Skip wells for samples that did not produce any colonies. Keep the toothpick in the well to track which colonies have been picked.
4) Remove toothpicks and cover block with air pore strip.
5) Place blocks in one layer in 37C incubator, no shaking. Do not stack blocks. Incubate overnight.
6) The following morning take out blocks. Check for growth, write down if some samples did not grow.
7) Make glycerol plate copy of blocks to keep at TIGR. Write lib_id on 2 sides of freezer plate. Check orientation of plate.
8) Pipette 30 µL 50% glycerol into freezer plate.
9) Pipette 120 µL culture into freezer plate. Pipette up and down a few times to mix.
10) Freeze TIGR glycerol copy at -80C. Do NOT freeze deep well block. Prepare for Agro mating immediately.

Tri-parental Mating with Agrobacterium

Supplies

- Agrobacterium colonies (GV3101)
- pRK E. coli helper strain colonies
- LR clone E. coli colonies
- LB media
- LB agair plates (4 chamber plates, VWR #62407-071)
- LB agar plates with rifampicin (50 µg/mL)+ gentamicin (50 µg/mL) + kanamycin (50 µg/mL) (4 chamber plates, VWR #62407-071)
- Deep well block, air pore strip, sterile flasks

Timeline

Day 1	Day 2	Day 3	Day 4	Day 5
Start liquid cultures: Agro GV3101 E. coli pRK helper E. coli LR clones	Plate 3 strains together on LB Agar	Streak loopful on LB rif + gent + kan, grow 2 days at 28C	Grow plate	Colonies ready

Mating procedure

1) Start 50 mL culture of Agrobacterium GV3101. Grow in LB rifampicin (50 µg/mL)+ gentamicin (50 µg/mL). Grow overnight at 28C, 250 rpm

2) Start 50 mL culture of pRK E. coli helper strain. Grow in LB Kan (50 µg/mL). Grow overnight at 37C, 250 rpm.

3) Start 250 µL colony cultures (described in "E. coli Transformation with LR reactions" section). Grow overnight at 37C, no shaking.

4) The following day, plate 50 µL Agro culture, 50 µL pRK E. coli helper strain, and 50 µL E. coli LR clone culture together on LB agar plate. This is the mating. Do one mating reaction for each LR clone.

5) Incubate LB Agar plates overnight at 28C.

6) The next day, there should be a bacterial lawn. There are two options for plating:

    a. Take a small loopful and streak it on LB rifampicin (50 µg/mL)+ gentamicin (50 µg/mL) + kanamycin (50 µg/mL). This will select for Agro with the pYXT2 construct.
    b. Set up deep well block with 400 µL LB/well. Take toothpick and touch bacterial lawn. Once all samples have been picked, cover block with seal and *lightly* vortex to disperse cells. Plate out 30 µL cells on LB rifampicin (50 µg/mL)+ gentamicin (50 µg/mL) + kanamycin (50 µg/mL).

7) Incubate 2 days at 28C. This should produce a lot of colonies.

8) Take pictures with epi-light and save images.

9) Store colonies in plastic bag or seal plates with parafilm and store at 4C until colony picking.

Agrobacterium Colony Screen

Supplies

- LB media with rifampicin (50 µg/mL)+ gentamicin (50 µg/mL) + kanamycin (50 µg/mL)
- Toothpicks
- 2X master mix (Promega #M7505)
- 20 mM NaOH
- Promoter specific primers
- PCR plate
- 96 well freezer plates for glycerol stocks
- 50% glycerol
- Reservoirs, multi-channel pipettes, tips, foil seals or heat seals, etc.

Pick colonies

1) Prepare 2 deep well blocks with 250 µL LB media with rifampicin (50 µg/mL)+ gentamicin (50 µg/mL) + kanamycin (50 µg/mL).
2) Pick colonies into 2 blocks: 1 colony/sample/block maintaining same well locations from primer plates.
3) Grow at 28C, 225 rpm for 2 days.
4) After 2 days, check growth. Track samples that did not grow.
5) Make glycerol stock of Agro cultures:
    a. Label freezer plate with lib_id
    b. Check plate orientation
    c. Pipette 30 µL 50% glycerol into wells
    d. Pipette 120 µL Agro culture into wells and mix by pipetting up and down
    e. Put freezer plate into -80C. Save deep well block for PCR.

PCR Conditions

1) Pipette 20 µL 20 mM NaOH into new PCR plate.
2) Add 3 µL Agro culture into PCR plate with NaOH.
3) Seal plate and incubate at 37C for 5 min.
4) Transfer 2 µL into new PCR plate and set up PCR reactions:

Reagent	**per rxn**		**96 rxns (plus 20%)**	
Milli-Q H2O	4.0	µL	460	µL
2X PCR mix	10	µL	1150	µL
Specific Primer mix 2.5 uM	4.0	µL	---	µL
Template	2.0	µL	---	µL
Pipette into each sample:	14 µL			
Total reaction volume:	20 µL			

Cycling conditions:
Program: <MAIN>,
calculated mode
1X: 95C 2 min
30X: 94C 30 sec, 55C 30 sec, 72C 3 min
1X: 72C 5 min
1X: 4C forever

Plant Transformations

Supplies

- Healthy Arabidopsis plants
- Lab tape and sharpie
- Agrobacterium strain carrying promoter reporter construct
- LB media plus rifampicin (50 µg/mL)+ gentamicin (50 µg/mL) + kanamycin (50 µg/mL)
- 5% sucrose solution (Fresh)
- 28C shaking incubator
- Silwet L-77

Transformation Procedure

Adapted from Simplified Arabidopsis Transformation Protocol (Clough and Bent)

1) Grow healthy Arabidopsis plants until they are flowering. Grow 3 plants/promoter reporter construct.

2) Clip first bolts to encourage proliferation of many secondary bolts. Plants will be ready roughly 4-6 days after clipping. Clipping can be repeated to delay plants. Optimal plants have many immature flower clusters and not many fertilized siliques, although a range of plant stages can be successfully transformed.

3) Prepare list of promoters to work with from the database and edit the transformation attempt and user field for these agro clones with attempt letter and user name.

4) labels for plants
    a. Use lab tape to label plant pot with plant number.
    b. Plant number scheme:
    Agro Plate#.Well.Attempt#.Transformation Plant #

5) Prepare *Agrobacterium tumefaciens* strain carrying promoter reporter construct. Use a 125 mL flask to grow a 50 mL culture in LB media + rifampicin (50 µg/mL)+ gentamicin (50 µg/mL) + kanamycin (50 µg/mL). Grow for 2 days @ 28^{o}C. You can use mid-log cells or a recently stationary culture for transformation.

6) Check OD_{600}. If OD is ~0.8, cells are ready, but OD can be higher or lower and still work.
7) Transfer culture to 50 mL falcon tube.
8) Spin down Agrobacterium culture at 6000 rpm, 4C for 5 min.
9) Resuspend to OD_{600} = 0.8 in 50 mL 5% Sucrose solution (if made fresh, no need to autoclave).

10) Before dipping, add Silwet L-77 to a concentration of 0.05% (500 µl/L) and mix well. If there are problems with Silwet L-77 toxicity, use 0.02% or as low as 0.005%.

11) Dip above-ground parts of plant in Agrobacterium solution for 2 to 3 seconds, with gentle agitation. You should then see a film of liquid coating plant. Some investigators dip inflorescence only, while others also dip rosette to hit the shorter axillary inflorescences.

12) Wrap dipped plants in the ara-cyc transparency sheets.

13) Water and grow plants normally in ara-cyc tubes. Stop watering as seeds become mature.

14) Harvest dry seed. Transformants are usually all independent, but are guaranteed to be independent if they come off of separate plants. Proceed to screening seed for kan resistance once dry seeds are obtained.

Screening T1 seeds for Kanamycin Resistance

Supplies

- 1N KOH (5.6 g KOH in 100 mL water)
- Seed selection media (see below)
- Seed sterilization solution (see below)
- Sterile dH2O
- Parafilm
- Sterile 0.1% agarose
- 150X15 mm Petri Dish (Falcon #1058)

Prepare Seed Selection plates

Prepare Seed selection media.

1) 1L:
    a. 4.3 g MS Salts
    b. 0.5 g MES
    c. Adjust pH to 5.7 with 1N KOH.
    d. Add 8 g phyto-agar.
2) Autoclave.
3) Add kanamycin (final: 50 µg/mL) and vanomycin (final: 500 µg/mL).
4) Pour into 150x15 mm plate in hood. Dry plates in hood.

Sterilize Seeds

1) Make seed sterilization solution (50% bleach, 0.02% Triton X-100)
    a. 100 mL:
        i. 50 ml of bleach
        ii. 20 ul of Triton X-100
        iii. 50 ml H2O
2) Make 0.1% agrose, sterile
3) Put seed in seed sterilization solution. Place on shaker for 7 minutes.
4) Rinse 3X in sterile dH2O
5) Resuspend in 8 mL sterile 0.1% agarose and pour onto large selection plates in even distribution.
6) Let sit in hood with lid set off for 10-15 minutes to allow water to evaporate.
7) Seal plates with parafilm.
8) Put in 4C for 2 days
9) Move plates to growth room on shelf.

Tracking

Label kanamycin screening plates with:

1) Date of plating
2) Agro Plate# - Well – Attempt # - Transformation Plant #

Planting, Growing, and PCR screening T1 plants

Supplies

- Soil
- Sheet of black plastic 6-cell trays in plant growth room
- 0.25 N NaOH
- 0.25 N HCl
- 0.5 M Tris pH8
- 0.25% nonidet P-40
- Shrimp Alkaline Phosphatase (GE Healthcare Life Sciences #E70092Z)
- Exonuclease I (GE Healthcare Life Sciences #E70073Z)

Tracking & Transplanting Seedlings

Once seedlings have grown on kanamycin plates in the growth room for 1-2 weeks, select the growing seedlings for transplanting into soil. Tweezers work well for this. Set small seedling directly on wet soil. Water with squirt bottle. Be careful when transplanting.
Pot size: each cell 5 cm x 5.5 cm. 1 plant/cell.

Label plants: **Agro Plate #.Well.Attempt #.Transformation Plant #.Plant #**

Leaf PCR

Once plants have developed a few young leaves, it is time to do leaf PCR.

Stock solutions (50 mL):

Stock	Components:
0.25N NaOH	0.5g NaOH
0.25N HCL	1.1 ml (36.5% HCL) 11.6N HCL + 48.9 ml H2O
0.5 M Tris (pH 8)	25 ml Tris (1M)
0.25% nonidet P-40	0.125ml nonidet P-40 +49.875ml H2O or 0.25% Igepal CA-630

Method:

1) Pick or cut off piece of leaf 1/2-1 cotyledon size (about 3-4 mm x 3-4 mm) and put in PCR plate or tube.
2) Add 40ul 0.25N NaOH. Boil for 30 seconds (98C on PCR machine).
3) Add 40ul 0.25N HCl.
4) Add 20ul 0.5M Tris 0.25% nonidet P-40 solution. Boil for 2 mins (98C on PCR machine).
5) Spin down, discard liquid.
6) Set up PCR reactions with leaf inside the tube.

Reagent	**per rxn**		**96 samples (plus 20%)**	
5x HF buffer	6	µl	690	µl
10 mM dNTPs	0.6	µl	69	µl
10 µM PC1 primer	2	µl	230	µl
10 µM PC2 primer	2	µl	230	µl
H2O	19	µl	2185	µl
Phusion DNA polymerase	0.4	µl	46	µl
Total	30	µl		

Cycling conditions:

1X: 98C, 30 sec
25X: 98C, 10 sec; 56C, 30 sec; 72C, 1:30 sec, X 25
1X: 72C, 10 min
1X: 4C, forever

Run 10 ul of sample on 1% gel to make sure PCR worked. Save remainder for SAP treatment and sequencing (next section).

Exo-SAP Treatment of PCR products

Purpose: The Exo-SAP treatment is used to clean-up PCR products of leftover primers and nucleotides which would interfere with sequencing reactions. SAP removes the phosphate group from the excess nucleotides and the exonuclease destroys the leftover primers.

1) Prepare master mix for clean-up of PCR reactions.

Reagent	**per rxn**		**96 samples (plus 20%)**	
SAP (1 U/µL)	0.5	µL	57.5	µL
Exonuclease I (10 U/µL)	0.1	µL	11.5	µL
SAP Buffer (10X)	0.5	µL	57.5	µL
dH2O	8.9	µL	1023.5	µL
PCR product	8.0	µL	----	
Total Volume	18	µL		

2) React samples in thermocycler with following conditions:

1X: 37C, 60 minutes
1X: 72C, 15 minutes
1X: 4C, forever

3) After reacting samples, spin them down, label the plate well with lib_id (for JTC) and place in -20C.

4) Send PCR products for sequencing with PC1 primer. Send 250 µL 5 pmol/µL primer.