From TAIR
AGROBACTERIUM COMPETENT CELLS

You should double up this protocol - it is almost the same amount of work and you can thus get some 80 tubes.

1. Inoculate colony O/N in 2 ml YEP + antibiotics at 28C shaker. ABI - 50 KAN & 25

Chlor, gv3101 - 25GEN 2. Transfer O/N culture to 200ml YEP in a sterile 500ml flask and shake at 250rpm until the OD is 0.3 (4-5hrs)

3. Spin in sterile 50ml screw cap tubes 4C 5krpm 10,. Check to make sure cells are pelleted, if not repeat at higher speed.

4. Aspirate supernatant, resuspend pellet in 20ml ice cold 1mM HEPES pH7 (sterile filtered), respin.

5. Repeat 4. two more times!

6. After aspirating, resuspend pellet in 2ml ice cold 10% glycerol (sterile filtered).

7, ASAP dipense in 40ul aliquots in pre-chilled, sterile eppis, freeze in lN2 and store -70C

AGROBACTERIUM ELECTROPORATION

Safety precautions

1. Never change any other settings than stated while unit is charging (this may damage both the electroporator and power supply)

2. Keep unit away from water in a dry area and away from flammable materials.

3. Never short circuit terminals.

4. Whilst delivering pulse, keep hands away from chamber and cuvette. The result may otherwise be shocking.

DNA preparations

DNA for electroporation must be free of salt, RNA or protein. DNA (in TE buffer) should be first treated with RNase, then twice extracted with phenol/chloroform.

This will remove protein and RNA. To remove salt, EtOH precipitate the DNA and wash twice with 70% ethanol. Resuspend the DNA at 0.4 -1

ug/ml.

Preparing the electroporator

There are two types of cuvettes 1 and 2mm. Most Agro protocols use 2mm (Invitrogen #650009 w/blue lids).

1. Make sure power supply is off.

2. With Charge/Pulse switch of the electroporator in the PULSE position, connect the leads from the power supply to the corresponding coloured terminals on the back of the electroporator.

3. Set Arm/Disarm dial to disarmed

4. Set Capacitance selector to 50 mF

5. Set Load resistance to 200W

6. Turn on power supply

7. Set maximum power to 25 W

8. Set current to 25 mA

9. Set voltage to 1800 V

10. Allow power supply to stabilize (still in pulse position)

11. Select the CHARGE position using the Charge/Pulse switch. After 20-30 seconds, the charging light will glow.

12. Check that voltage meter still reads 1800 V

13. Set Arm/Disarm to the ARMED position and the armed light will glow.

14. Switch the Charge/Pulse to the PULSE position. The pulse light will glow briefly and both the charging and armed lights will go out.

15. Set Arm/Disarm to the DISARMED position (the armed light should be off)

Electroporating

Electrocompetent bacterial cells, YEP media and DNA solutions must be kept on ice before mixing. Note that the following steps should be carried out in under 1' and that you should be wearing glasses and gloves

16. mix 1-2ml DNA (600 ng) with 40ml cells

17. Transfer the DNA/cell mixture to a cuvette on ice avoiding air bubbles by gently shaking the cuvette

18. Dry outside of the cuvette with tissue paper and insert the cuvette into the cuvette chamber with notch facing towards you

19. Close cuvette chamber lid

20. Set Arm/Disarm to ARM (arm light comes on)

21. Set Charge/Pulse to pulse and the pulse light will come on briefly

22. When pulse light is off, set Arm/Disarm to DISARM (arm light comes on) and remove cuvette

23. With DNA/agro mix still in cuvette, add 500ml cold YEP (no antibiotics) and mix solution by gently pitppeting up and down

24. Transfer the cells to an eppi and incubate at 28C for 2-4 hour

25. Leave the electroporator with the switch in the PULSE position

26. Plate 200ml on YEP + antibiotics

27. Incubate at 28C and colonies will appear in 2-3 days

Re-using cuvettes

Fill a used cuvette w/ 0.1M H2SO4 and let stand 15'. Rinse 6x w/ dH20, then 2x w/ 96% EtOH. Store them well-covered in 70% EtOH

AGROBACTERIUM MINIPREP

Agros to be used for plant transformation should be checked for the presence of the Ti plasmid as plant transformation and the analysis of
transgenic plants is time consuming. The easiest way to do this is to make an agro miniprep and to use PCR to determine that the cells contain
your construct. PCR is necessary here because the Ti plasmid is single copy and you can barely see it on agarose gels.

1. Grow cells overnight in 5 ml LB or YEP with antibiotics.

For pMONs in ABI - 50ug/ml KAN, 50ug/ml Spec, 25ug/ml Chlor

For pBI types in gv3101 - 50ug/ml KAN, 25ug/ml GEN

2. Remove 1 ml cells to two microfuge tube

3. Centrifuge 45 sec and remove the supernatant with aspiration

4. Add 1 ml cells more to both tubes and repeat step 3

5. Vortex the pellet, add 100 l MPS1 solution, vortex again and incubate the tubes at room temperature for 5 min

6. Add 20 l of a 20 mg/ml lysozyme solution, vortex-spin 1 sec and incubate 15 min at 37C.

7. Add 200 l MPS2 solution(freshly made), mix gently by turning the rack 3-4 times and incubate 5 min on ice

8. Add150 l MPS3, vortex for at least 10 sec and incubate 5 min on ice

9. Centrifuge for 5 min and remove the supernatant to new tubes

10. Add 400 l phenol/chloroform/isoamyl alcohol (25:24:1), vortex, centrifuge for 5 min and remove the supernatant to new tubes

11. Repeat step 10

12. Repeat this step with chloroform alone

13. Add 300 l isopropanol and incubate on ice for 10 min

14. Centrifuge for 5 min and wash pellet with 70 % EtOH

15. Dry pellet and resuspend the two tubes in a total of 50 l TE-buffer+RNase, use 2ml for a PCR, freeze the rest.

MPS1 for 50 ml Stock

50 mM glucose	1M	2,5 ml
10 mM EDTA	0,5mM	1 ml
25 mM Tris pH=8.0	1M	1,25 ml

MPS2 for 10 ml

0,2 N NaOH 10N 200 ml

1% SDS 10% 1 ml

H2O 8,8 ml

MPS3 for 100 ml

5 M potassium acetate 60 ml

glacial acetic acid 11,5 ml

H2O 28,5ml

AGROBACTERIUM PROTEASE K MINIPREP (PHENOL FREE)

1) Resuspend pellet from 3ml cultures in 200uls STET

2) Add 10ul Lysozyme/RNase solution and incubate for15min at 37 C.

3) Add 1ul 10mg/ml Protease K solution and incubate at 50 C for 15min.

4) Incubate at 94 C 1min then vortex vigourously 30 sec.

5) Remove gooey supernatant to new tube and add 5uls 5% CTAB. Wait 5min then spin 5min.

6) Remove supernatant and rususpend in 300uls 1.25M NaCl by vigourous vortexing. Spin tubes for 1min and transfer supernatant to new tube
with 750uls 96% EtOH + 1mM PMSF. 5min RT, 30min spin.

7) Wash pellet in 70% EtOH+ 1mM PMSF, dry and resuspend in 40uls TE 8.0

Can visualize 10uls in a restriction digest.

ARABIDOPSIS TRANSFORMATION BY VACUUM INFILTRATION

This protocol is modified from Bechtold, Ellis and Pelletier (1993). "In planta Agrobacterium mediated gene transfer by infiltration of adult
Arabidopsis thaliana plants". [C.R. Acad. Sci. Paris, Life Sciences 316: 1194-1199].

PLANT GROWTH:

1. Take seeds with a brush and place them into 8cm square pots filled with soil. Don't compress the soil too much and water the pots thoroughly
with 2-3 pot-vol to remove excess nutrients. Place 12-16 seeds in each pot.

Place the pots in the cold room for two days before transfering them to the growth chamber. Grow the plants for three weeks in short days (10 hr
or less) to get large plants and a greater seed yield. Transfer the pots to long days to induce bolting. Grow plants to a stage at which bolts are
around 10 cm tall.

2. Clip off emerging bolts close to rosette leaves to encourage growth of multiple secondary bolts. Infiltration will be done 7 to 9 days after
clipping (plants will be 10-15 cm high and the biggest of the inflorescens will have made the first tiny silique. Do not water the plants the day
before vacuum infiltration.

VACUUM INFILTRATION:

3. Start a 4ml agrobacterium culture (YEP+antibiotics) inoculated from a -800C stock or from a plate. Grow cells O/N to 48h depending on the
strain. Add this culture to 250 ml of YEP+antibiotics (A 250ml culture will give enough cells for infiltration of 6 pots). Grow the culture between
O/N and 2 days (depending on the strain) to OD600 = 1.2-1.8. The culture will have a mother of pearl appearance (not lumpy or black).

4. Spin down agros at 5000rpm for 10 min in 250ml centrifuge bottles, resuspend in infiltration media to an OD600 = 0.8 in a minimum volume
of 300ml.

5. Poor the agro suspension into a beaker of an appropiate size (400ml is ok). Place the beaker into the vacuum jar. Degass the solution by
drawing vacuum until bubles form. Place a paper towel under the beaker to avoid that the beaker gets stuck in the bottom of the vacuum jar.

6. Sprinkle the plants with water 5 min prior to infiltration (optional) and then invert plants into the culture solution. Make sure that all the flowers
are submerged and leave 2cm between the rosettes leaves and the agro suspension. Don't let the culture contact the rosette or soil as this could kill
the plants. Avoid that the solution boils over when you pull the vacuum. Make sure that the soil is only moist, so that the water from the pots does
not enter into the culture suspension (therefore we recommend not to water the plants the day before infiltation). Draw vacuum for 15-20 min for
WS and 30 min for Col-0 at a pressure close to 0.05 Bar (we are using an oil pump).

7. Before removing the plants from the vacuum jar place a plastic bag over the pot and beaker. Pull out and remove plants from the beaker, lay

pots on their side (to avoid that excess infiltration media runs down into the soil). Fold over the top of the plastic bag and staple them twice. The other possibility is to place the pots laying on their side into a tray and cover the whole box with saranwrap. Put them in a growth chamber for one night. Next day move them to the green house. Put the plants in vertical position and open the bags. Next day get rid off the bags. In case you have the plants in trays: put also the plants in vertical position and use sticks and saranwrap to make a kind of a tend around the plants. Next day remove the plastic. In hot summers, we recommend to give plants a shower after we have placed them in vertical position (the purpose of this is to remove the sugars from the infiltration media which decrease fungal infection).

8. Grow plants for approx. four weeks, keeping bolts from each pot together but separated from neighbouring pots

9. When the siliques begin to turn yellow, place the pot on its side with the plants inside a big envelope. Leave them for one week to dry out and cut off the plants. Let the seeds dry in the envelope and clean them 10 days later (keep all the seeds from one pot together). Store the seeds in the cold room for one week before plating them.

KANAMYCIN SELECTION PROTOCOL

1. Sterilisation of seeds:

aliquot seeds in 15ml falcon tubes (approx 700 seeds/tube, you can estimate the ammount of seeds by first drawing a square plate of 9cmx9cm on a paper and spreading the seeds on it). Add 10 ml of hypoclorite solution. Shake tubes for 10 min. Remove the solution and add 10ml of 70% ethanol. Wait 2 minutes. Discard EtOH and wash seeds 2-3 times with 10ml of sterile water.

Resuspend seeds with 8ml 0.7% top agar (no warmer than 55oC). 2. Spread seeds onto selection plates (MS+Kan). Dry plates in laminar flow hood until the top agar has solidified.

3. Vernalize plates for two nights in the cold room at 4oC. Transfer the plates to the growth chamber (21oC with continous light).

4. After aprox 7 days transformants should be clearly identifiable as dark green plants with healthy green secondary leaves and roots that extend into the selective medium. Root growth is the most clear maker to identify transformants at an early stages.

To make sure that the transformants are positive transfer them to a new MS+Kan plate and leave them there for a few days (if they turn yellow is

because they are faulse positives). Transfer the seedlings to soil.

If you have contamination on your plates at this step, transfer the transformants as early as possible to soil.

5. Grow the plants and collect the seeds.

Infiltration Media

1/2 x Murashige&Skoog salts (SIGMA #5524)

1X B5 vitamines (1ml of 1000x stock) (SIGMA; #G-2519) Gamborg's vitamine powder, to prepare the 1000x stock disolve 11.2g in 100ml
water.

5% sucrose

adjust to pH 5.7 before autoclaving

after autoclaving add:

- Benzylamino Purine (BAP), 10 μl per liter of a 1 mg/ml stock in DMSO. By adding the hormone just before use, you can keep infiltration
media as a stock for at least one week prior to infiltration.

- we recommend to add 0.01% silwet to the infiltration media to increase transformation efficiency especially for Landsberg and colombia
ecotypes. (silwet is from LEHLE SEEDS, cat no VIS-01 VAC-IN-STUFF (silwet L-77)

Selection plates:

1x Murashige&Skoog salts

1% sucrose

adjust pH 5.7 with 1M KOH.

0.7% Difco agar.

autoclave, cool, and add:

1x MS vitamines (SIGMA #M-7150). Take 1ml of 1000x stock prepared by disolving 10.3gr in 100ml of water.

antibiotic (kanamycin 50mg/l).

Top agar:

1x Murashige&Skoog salts.

1% sucrose.

adjust pH 5.7 with 1M KOH.

0.7% Difco agar.

autoclave.

before use: boil in the microwave and keep in water bath at 50-550C.

YEP media (liquid):

10 g /l Bacto peptone (Difco)

10 g/l Yeast extract (Difco)

5 g /l NaCl

For YEP plates add 15gr/l Difco bacto agar.

Hypoclorite solution:

for 50 ml:

4ml Na Hypoclorite 15%

255l Tween-20

water to 50ml