

Cellulase and Macerozyme-PEG-mediated Transformation of Moss Protoplasts

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[Abstract] This protocol describes the generation of protoplasts from protonemal tissue of the moss *Physcomitrium patens* (syn. *Physcomitrella patens*), using Cellulase ONOZUKA R10 and Macerozyme R10, followed by polyethylene glycol (PEG) mediated transformation. The protonemal tissue grown in liquid suspension was harvested and treated with enzyme cocktails mix of 1.5% Cellulase ONOZUKA R10 and 0.5% Macerozyme R10 to generate 1,8 million protoplasts within 3 h.

Keywords: Moss, *Physcomitrium patens* (syn. *Physcomitrella patens*), Protoplastation, Cellulase, Macerozyme

[Background] Thanks to its efficiency in the natural homology directed repair *Physcomitrium patens* is ideal to use as a plant experimental system, especially for heterologous expression of genes, permitting the generation of transformants with predictable properties and characteristics. Moreover, *P. patens* has the advantage of low-cost cultivation and it can be maintained in large quantities in bioreactors (Reski *et al.*, 2018). The genetic engineering of *P. patens* cells allows the large-scale production of natural products and drugs such as thapsigargin and other terpenoid drug candidates, which are difficult of access (Simonsen *et al.*, 2009). This protocol shows how to isolate and transform *P. patens* protoplasts released from the haploid protonema filaments. *P. patens* has been already used for generation and PEG-mediated transformation of protoplasts. In the previous experiments protoplastation on *P. patens* is performed with the enzyme Driselase from Basidiomycetes (Cove *et al.*, 2009; Bach *et al.*, 2014). With the model plant *Arabidopsis thaliana*, 60 min of incubation with constant shaking in darkness allows one to obtain a consistent amount of viable protoplasts (Fesenko *et al.*, 2016). Although the *Physcomitrium* tissue digestion fulfilled by Driselase is highly efficient and fast (Bach *et al.*, 2014; King *et al.*, 2016), the instable delivery and availability of Driselase led to the study of a different procedure for protoplastation and to look for more widely used enzymes. Here we describe an alternative efficient method, using the enzyme Cellulase ONOZUKA R-10 in combination with Macerozyme R-10. Cellulases and pectinases have been successfully tested on different plants such as *Fritillaria imperialis* L. (Chamani and Tahami, 2016), *Penium margaritaceum* (Raimundo *et al.*, 2018), *Ananas comosus* L. (Priyadarshani *et al.*, 2018), *Brassica oleracea* L. and *Brassica napus* L. (Kaur *et al.*, 2018) and it is regularly used on *Arabidopsis thaliana* (Abel and Theologis, 1994; Yoo *et al.*, 2007), but to our knowledge it has not been published on *P. patens*. Additionally, in this protocol the incubation is performed without shaking. Further modifications of the experimental conditions could possibly optimize

the protocol even more to yield more protoplasts.

Materials and Reagents

1. Falcon™ (Size 50 ml; D-51588)
2. Cell strainers (70 µm nylon mesh, Fischer brand, catalog number: 22363548)
3. Cell strainers (40 µm nylon mesh, Fischer brand, catalog number: 22363547)
4. Round bottomed glass tube with screw cap (size: 11 ml; Pyrex, catalog number: 10004654)
5. Scalpel blade (Thermo Scientific™ 53221, catalog number: 12657776)
6. Petri-dishes (Fisherbrand™ Polystyrene Petri Dishes, Sterile; size: 90 mm)
7. Syringe filter (0.45 µm, FRISENETTE, catalog number: CALS2504100S)
8. Sterile syringe (20 ml and 50 ml) (Chirana, catalog numbers: CH03020L; CH03050L)
9. Sterile pipette tips (Labsolute, non-pyrogenic, single use, size: 50 µl, 10 µl, 5 µl)
10. Moss (*Physcomitrella patens*) that is blended 4-5 days in advance (liquid culture) and grown at 16 h light/8 h dark cycle at 25 °C in presence of 5 mM of Ammonium Tartrate as described previously in Bach *et al.* (2014).
11. Cellulase ONOZUKA R-10 1.5% (w/v) (DUCHEFA, catalog number: C8001.0010, CAS No: 9012-54-8), storage temperature 2-8 °C
12. Macerozyme R-10 0.5% (w/v) (DUCHEFA, catalog number: M8002.0010, CAS No: 9032-75-1), storage temperature 2-8 °C
13. D-Mannitol (Duchefa Biochemie, catalog number: M0803.5000; CAS No: 69-65-8)
14. CaCl₂ (SIGMA, catalog number: C3306-550G; CAS number: 10035-04-8)
15. MES hydrate (SIGMA, catalog number: M2933-1Kg)
16. KOH (SIGMA, catalog number: 484016-1KG; CAS No: 1310-58-3)
17. Trizma base (SIGMA, catalog number: T6066-1Kg; CAS no: 77-86-1)
18. HCl (VWR Chemicals, catalog number: 20255.200; CAS no: 7647-01-0)
19. Ca(NO₃)₂·4H₂O (SIGMA, catalog number: C2788-500G; CAS no: 13477-34-4)
20. Polyethylene glycol (PEG, MW 6000) (SIGMA, catalog number: 81253-250G; CAS no: 25322-68-3)
21. MgCl₂ (SIGMA, catalog number: M8266; CAS no: 7786-30-3)
22. Agar, plant cell culture tested (Alfa Aesar, catalog number: H26724; CAS no: 9002-18-0)
23. Ammonium tartrate (SIGMA, catalog number: 09985-250G; CAS No: 3164-29-2)
24. H₃BO₃
25. MnCl₂·4H₂O
26. AlK(SO₄)₂·12H₂O
27. CoCl₂·6H₂O
28. CuSO₄·5H₂O
29. ZnSO₄·7H₂O
30. KBr

31. KI
32. LiCl
33. SnCl₂·2H₂O
34. 8.5% Mannitol (see Recipes)
35. 1 M CaCl₂ (see Recipes)
36. 2 M KOH (see Recipes)
37. 0.2 M MES pH 5.8 (see Recipes)
38. Protoplast media (PM) (see Recipes)
39. Protoplast wash solution (PW) (see Recipes)
40. 0.4 M Tris-Cl pH 8.0 (see Recipes)
41. PEG-MCT (see Recipes)
42. 1 M MgCl₂ (see Recipes)
43. MMM solution (see Recipes)
44. PRMB media (see Recipes)
45. PRMT media (see Recipes)
46. BCD Media (see Recipes)
47. BCD Plate (see Recipes)
48. 500 mM Ammonium tartrate (see Recipes)

Equipment

1. Flasks, Erlenmeyer (1 L, sterile)
2. Reagent bottles (500 ml, 250 ml, 100 ml)
3. Serological pipette (Brand® Accu-jet® pro, catalog number: 19B17038)
4. Centrifuge (ThermoFisher, model: Heraeus™ Multifuge™ X1R)
5. Haemocytometer (Neubauer-improved 0.1 mm, Superior MARIENFELD Germany; catalog number: 0650030)
6. Light microscope (OLYMPUS, BX40)
7. 30 °C incubator (Gerhardt Thermoshake)
8. Scalpel handle (Thermo Scientific™ 53410, catalog number: 12667776)
9. Forceps (Bochem™ 1020R, catalog number: 10333511)
10. Microwave (Whirlpool, GUSTO: GT285)
11. Sterile cellophane disks (Size: 80 mm, Company: Focus Packaging & Design Ltd, PKS150 70-01, Batch: 05377)
12. Surgical tape (Micropore, 1,25 cm X 9.1 m, ½ in x 10 yd, Ref: 1530-0)
13. Bunsen burner (Campingaz, Easy clic™ Plus; CV300 Plus)
14. Laminar Air Flow Bench (LAF Bench)
15. Water bath at 45 °C (MgW LAUDA MS)
16. Rocker (Brand: Desaga, Starstedt-Gruppe)

17. Homogeniser (IKA® T25 digital ULTRA TURRAX)

Procedure

Procedural guidelines

Notes:

1. Perform all steps at room temperature (20-25 °C) unless noted otherwise.
2. Use solutions and equipment sterilized by autoclavation or filter sterilization.
3. Always use microbiological aseptic techniques and work in a sterile facility when handling moss.
4. Perform all centrifugation steps at 200 x g with slow acceleration and breaking speeds* unless noted otherwise.

*Acceleration speed 2 and deceleration speed 4 when using the Heraeus™ Multifuge™ X1R (Thermo Fisher).

Protoplasts generation

A. Moss preparation

Note: Blend the moss twice before use, with at least 7 days gap between each blend. Add 5 mM of ammonium tartrate to grow the moss. After the second blend, use the moss on either 4th or 5th day for transformation.

1. Filter 5-day old protonemal moss tissue by straining through 70 µm cell strainer.
2. Place the cell strainer on a 50 ml Falcon tube. Filter 40 ml of moss culture through the strainer to collect the moss on top (Figure 1). Transfer the cell strainer onto a new Falcon tube and repeat the filtration step multiple times to collect ~1.5 g of moss.
3. Transfer the cell strainer with collected moss on a fresh 50 ml Falcon tube. Pass 50 ml of autoclaved demineralized water through the strainer to wash and remove the traces of BCD media from the moss. Repeat this washing step 4-5 times.
4. Split the collected moss from the strainer into five 50 ml Falcon tubes using a sterile forcep. Each tube should contain approximately 200 to 300 mg of vegetal tissue.

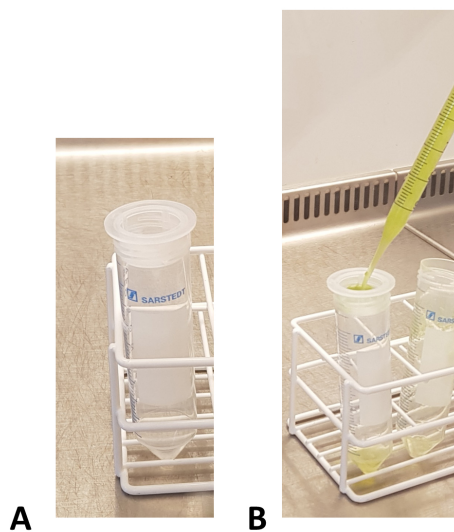


Figure 1. Collection of moss protonemal tissue for protoplastation. A. Sterile 70 µm cell strainer placed on a Falcon tube. B. Passing moss culture through the strainer to collect the moss on top.

B. Enzyme solution (prepare fresh)

1. Prepare enzyme solution in a glass beaker by adding 750 mg of Cellulase Onozuka R-10 to 50 ml of PM media. Stir using magnetic bead to dissolve the salt, until the solution looks clear.
2. Once the Cellulase completely dissolves, add 250 mg of Macerozyme R-10 and dissolve completely as before.

Note: The systematic addition of both salts ensures their complete dissolution. The enzyme solution at this point should have a light brown color.

3. Transfer the enzyme solution to a 50 ml Falcon tube and centrifuge at 5,000 g for 2 min (set the acceleration and deceleration speed of centrifuge to 9).
4. Avoid the pellet and filter sterilize the supernatant by using 0.45 µm syringe filter into a new 50 ml Falcon tube, inside the LAF bench.
5. Divide 10 ml of sterile enzyme solution to five Falcon tubes containing moss (from Step A4), shake to suspend and transfer the mixture to five sterile Petri-dishes.
6. Incubate the protonemal tissue in the enzyme solution for at least 3 h at 30 °C in dark. After 1.5 h, microscopically assess the number of free-floating protoplasts in a drop of digested solution, followed by assessing after every 30 min. Proceed when most of the protonemal tissue has been digested.

IMPORTANT! Moss protoplasts are very fragile and can easily be damaged by disturbances. When handling protoplasts, proceed with extreme caution and avoid physical disruption. Use pipette tips with a wide opening (cut the tip of regular pipette tips with a heated sterile scalpel) and draw up protoplast suspensions slowly to avoid disruption caused by suction. Never vortex protoplast suspensions. Perform all steps under sterile conditions inside a LAF Bench.

C. Isolate protoplasts

1. Recover the protoplasts by filtering the suspension through a 70 μm cell strainer. Wash the Petri-dishes with 2-3 ml of PM to dissolve any leftover moss sticking to the surface, and filter this as well through the mesh, to collect all protoplasts.
2. Again, pass the digested solution through 40 μm mesh to further remove cell debris and undigested protonema.
3. Collect the filtrate in 50 ml Falcon tubes and distribute to five 10 ml round bottomed glass tubes.
Note: Use additional glass tube with 10 ml water to balance while centrifugation.
4. Centrifuge the protoplast suspension for 10 min at 200 x g, 22 °C.
5. Carefully decant the supernatant using a pipette rather than inverting the tube. This avoids the risk of losing the pellet as protoplasts pellet is not tightly adhered to walls of tube. While pipetting leave behind small volume of supernatant so that the protoplast pellet remains immersed.
6. Resuspend the protoplast pellet in approximately 1 ml PW solution by gently shaking the tube. Collect the protoplast suspensions from all tubes into one and add PW to obtain a total volume of 10 ml.
7. Repeat Steps C4 and C5.
8. Resuspend the protoplast pellet in 3 ml PW solution by gently shaking the tube. Estimate the protoplast density with a Haemocytometer. Count the number of protoplasts on both grid areas (see Figure 2 for an image of protoplasts) and use the average to estimate the number of protoplasts.

TOTAL VOLUME OF THE GRID AREA ON THE HEMOCYTOMETER:

$$1 \text{ mm} \times 1 \text{ mm} \times 0.1 \text{ mm} = 0.1 \text{ mm}^3$$

ESTIMATED NUMBER OF PROTOPLASTS IN THE SOLUTION:

$$\text{Protoplasts on grid area} \times 10^4 \times \text{total volume in ml} = \text{number of protoplasts}$$

9. Repeat Step C4.
10. Carefully decant most of the supernatant using a pipette and stop pipetting when the pellet starts to dissolve.
11. Resuspend the protoplast pellet in required amount of MMM solution to obtain a final concentration of 1.6×10^6 protoplasts/ml.

FINAL VOLUME MMM-PROTOPLASTS SUSPENSION:

$$\frac{\text{number of protoplasts}}{1.6 \times 10^6} = \text{final volume in mL}$$

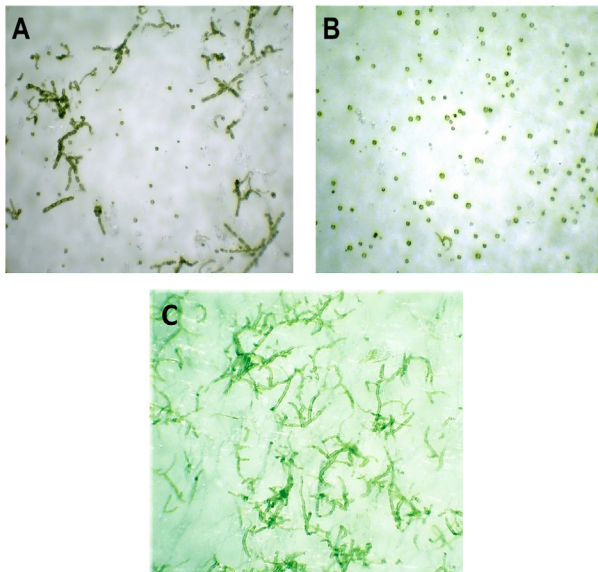


Figure 2. The figure show microscope images of protoplasts from *P. patens*. A. This is some free protoplast and with protonemal tissue. B. Only free protoplast. B is the ideal situation after the treatment. C. Undigested protonema tissue.

D. Protoplast transformation

1. For each transformation, transfer 20 μg of DNA to the bottom of a sterile 50 ml Falcon tube.

Note: Use 20 μg DNA in a volume not greater than 30-50 μl .

STEPS 2-6 ARE TIME SENSITIVE

2. Add 300 μl of protoplast suspension (from Step C11) and 300 μl of sterile PEG-MCT solution to the DNA and mix by gently tapping the tube.
3. Incubate the mixture in a water bath at 45 $^{\circ}\text{C}$ for 5 min, followed by 5 min incubation at room temperature. Meanwhile melt PRMT medium using a microwave. Keep the melted PRMT in a water bath at 45 $^{\circ}\text{C}$.
4. Add 300 μl PW solution to the "protoplast-PEG-DNA suspension" five times, with the interval of 1 min between each dilution.
5. Now add 1 ml PW solution five times with the interval of 1 min between each dilution.
6. Centrifuge the transformed protoplasts for 3 min at 200 x *g*. Carefully decant the supernatant using a pipette. At this point, the protoplast pellet may not be visible, so leave a little amount (~300 μl) of supernatant back while pipetting.
7. Resuspend the protoplasts in 500 μl PW solution.
8. Add 10 mM CaCl_2 to the warm PRMT medium (maintained at 45 $^{\circ}\text{C}$). Now add 2.5 ml of this CaCl_2 -PRMT mixture to the protoplast suspension and tap the Falcon tube to mix gently.
9. Distribute 1 ml of this mixture among three PRMB plates covered with single sterile cellophane disc.

Note: Slowly distribute the protoplast suspension on PRMT plate, while simultaneously and

gently spreading the mixture with your pipette tip as you proceed. This assures a uniform distribution and avoids the dome shaped gel blotch on the plate (cut the end of the 1,000 μ l tip with a heated scalpel for this step).

10. Let the protoplasts recover overnight in the dark at 25 °C.

Note: To mimic dark conditions, cover each plate with aluminum foil.

11. Incubate the plates in continuous light at 25 °C for 5 days.

12. Transfer the cellophane discs with protoplast containing PRMT-layer onto antibiotic containing BCD plates. Cultivate the moss under standard growth conditions (16 h day/8 h night cycle) at 25 °C and proceed with the selection of positive transformants.

Data analysis

Results

Estimated number of protoplasts in 3 ml of PW solution:

Protoplast on Grid 1 and Grid 2 and their average

$$\frac{\text{Grid 1} + \text{Grid 2}}{2} = \frac{41 + 78}{2} = 59.5$$

Protoplasts on grid area $\times 10^4 \times$ total volume in ml = number of protoplasts

$$59.5 \times 10^4 \times 3 = 178.5 \times 10^4 \text{ or } \mathbf{1.79 \times 10^6 \text{ protoplasts in 3 ml}}$$

Final volume MMM protoplast suspension:

$$\frac{\text{number of protoplasts}}{1.6 \times 10^6} = \frac{1.79 \times 10^6}{1.6 \times 10^6} = 1.118 \text{ ml}$$

Selection procedure to obtain stable moss lines

1. After 2 weeks on antibiotic containing BCD plates, transfer the cellophane disc with recovered transformants to fresh antibiotic containing BCD plates and incubate for another 2 weeks at 25 °C under standard growth conditions.

2. Transfer each colony to antibiotic free BCD plates (overlaid with single sterile cellophane). Incubate the plates at 25°C under standard growth conditions for 10 days.

Note: Transfer each colony individually on the BCD plate to avoid getting chimeric colony. Sterilize the forcep with 70% ethanol and fire each time after transfer.

3. After 10 days of incubation on selection free plates, transfer a small part of each protonemal colony to BCD plate with appropriate antibiotic. Incubate the plates for 10 days at 25 °C under standard growth conditions. Only the stable transformants grow on selection plates.

Note: Transferred protonema should only be collected from the growing end of each colony. Avoid transferring the whole colony or gametophores.

4. After 10 days of incubation on selection plates, transfer the cellophane on antibiotic free

selection plate and let grow for a week before individually transferring them to fresh BCD plates overlaid with cellophane.

5. Verify the transformants using genotype sequence verification, metabolic profiling, or proteomics or phenomics *etc.*

Recipes

1. 8.5 % (w/v) Mannitol (Prepare in advance)
 - a. Prepare 500 ml 8.5% (w/v) mannitol solution by adding 91.08 g of D-mannitol to 400 ml of demineralized water
 - b. After dissolving, add demineralized water to make the volume up to 500 ml and autoclave
2. 1 M CaCl₂ (prepare in advance)
 - a. Prepare 500 ml of 1 M CaCl₂·2H₂O by adding 73.51 g of CaCl₂ to 300 ml of demineralized water
 - b. After dissolving, add demineralized water to make up the volume to 500 ml
 - c. Do not autoclave, instead filter sterilize prior to use
3. 2 M KOH (prepare in advance)
 - a. Prepare 100 ml of 2 M KOH by adding 11.2 g of KOH to 50 ml of demineralized water
 - b. After complete dissolution of salt, add demineralized water to make up the volume to 100 ml and autoclave
4. 0.2 M MES pH 5.8 (prepare in advance)

Storage at room temperature

 - a. Prepare 100 ml of 0.2 M 2-(N-morpholino) ethylanesulfonic acid (MES) by adding 3.90 g of MES hydrate to approx. 40 ml of demineralized water, dissolve completely on magnetic stirrer
 - b. Bring the pH to 5.8 using 2 M KOH
 - c. Once the pH is stable, add demineralized water to make the volume to 100 ml
5. Protoplast media (PM) (prepared in advance)

Storage at room temperature

 - a. Prepare 100 ml of PM by adding 10 ml of 0.2 M MES (pH 5.8) and 1 ml of 1 M CaCl₂ to 89 ml of 8.5% mannitol solution
 - b. The working concentration of MES (pH 5.8) in PM is 20 mM and of CaCl₂ is 10 mM
 - c. Filter sterilize the media
6. Protoplast wash solution (PW) (prepare in advance)

Storage at room temperature

 - a. Prepare 100 ml of PW by adding 2 ml of 0.2 M MES (pH 5.8) and 1 ml of 1 M CaCl₂ to 97 ml of 8.5% mannitol
 - b. The working concentration of MES (pH 5.8) in PW is 4 mM and of CaCl₂ is 10 mM
 - c. Filter sterilize the media

7. 0.4 M Tris-Cl pH 8.0 (prepare in advance)
 - a. Prepare 100 ml of 0.4 M Tris-Cl by adding 4.84 g of Tris base to 70 ml of demineralized water
 - b. When dissolved, adjust pH to 8.0 with 2 M HCl and add demineralized water to make the volume up to 100 ml
8. PEG-MCT (prepare fresh)
 - a. Prepare 5 ml of PEG-MCT by adding 118 mg of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 125 μl 0.4 M Tris-Cl (pH 8.0) to 5 ml of 8.5% mannitol solution and dissolve
Note: Prepare 300 μl PEG-MCT solution per transformation.
 - b. Add 2 g of polyethylene glycol (PEG, MW 6000) and mix well
 - c. Keep the solution on a rocker for at least 2 h to completely dissolve the PEG and avoid recrystallization
 - d. Filter sterilize the PEG-MCT solution after the PEG is dissolved
Note: Here, PEG stands for polyethylene glycol and MCT stands for Mannitol+Calcium nitrate+Tris-Cl (salts used to prepare PEG solution).
9. 1 M MgCl_2 (prepare in advance)
 - a. Prepare 100 ml of 1 M MgCl_2 by adding 9.52 g of MgCl_2 salt to 80 ml of demineralized water
 - b. After dissolving, add demineralized water to make the volume up to 100 ml and autoclave
10. MMM solution (prepare Fresh)
 - a. Prepare 10 ml of MMM solution by adding 150 μl of 1 M MgCl_2 and 100 μl of 0.2 M MES to 10 ml of 8.5% mannitol solution in demineralized water. This is sufficient for 10-15 transformations
 - b. Filter sterilize prior to use
Note: Here, MMM stands for Mannitol + MgCl_2 + MES; PRMB stands for Protoplast Regeneration Medium Base layer; PRMT stands for Protoplast Regeneration Medium Top layer.
11. PRMB media (prepare in advance)
 - a. Prepare BCD media with 6% (w/v) mannitol and 0.7% (w/v) Agar in demineralized water, followed by autoclavation
 - b. Once slightly cooled, add a final concentration of 10 mM CaCl_2 before pouring to the Petri-dishes. When solidified, overlay the medium with a sterile cellophane disk
 - c. Prepare 3 Petri-dishes with PRMB medium per transformation
12. PRMT media (prepare in advance)
 - a. Prepare 200 ml of BCD media with 8% (w/v) Mannitol and 0.4 % (w/v) agar in demineralized water, followed by autoclavation
 - b. Once slightly cooled, aliquot 50 ml media to four sterile 100 ml reagent bottles inside the LAF bench
Optional: One of the aliquots can be stored in 60 °C oven to keep warm. This media can be

used on the same day for transformation or can be stored for max 2 weeks in melted form.

- c. Aliquots can be stored at room temperature and when required can be heated prior to use (see Step D3)

Note: Heating of the media in the microwave should be carried out under the supervision of experienced person while following the safety guidelines.

13. BCD Media (prepare in advance)

- a. Prepare 1 L of BCD media by mixing:
 - 12.5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$
 - 10 ml "Solution B" (25 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)
 - 10 ml "Solution C" (25 g/L KH_2PO_4 , pH adjusted to 6.5 with 4 M KOH)
 - 10 ml "Solution D" (101 g/L KNO_3)
- b. To this add 1 ml of "Trace element solution" (614 mg H_3BO_3 , 389 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 110 mg $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 55 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 55 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 55 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 28 mg KBr, 28 mg KI, 28 mg LiCl, 28 mg and $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ per liter)

14. BCD Plate (prepare in advance)

- a. For every transformation, prepare 3 Petri-dishes with BCD medium containing 0.7% (w/v) agar and the antibiotic corresponding with the selection marker used for transformation
- b. Add a final concentration of 1 mM CaCl_2 before pouring into the Petri-dishes

15. 500 mM Ammonium tartrate (prepare in advance)

- a. Prepare 100 ml of 500 mM of Ammonium tartrate ($\text{C}_4\text{H}_{12}\text{N}_2\text{O}_6$) by adding 9.21 g ammonium tartrate salt to 80 ml of demineralized water
- b. When dissolved further add water to make up the volume to 100 ml
- c. Filter sterilize prior to use

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Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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