Protoplast isolation and PEG-mediated transformation V.2

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Adapted from Yoo S-D, Cho Y-H, & Sheen J (2007) Nature Protocols and Arndell T *et al* (2019) BMC Biotechnology. Originally designed for the isolation of Arabidopsis mesophyll protoplasts to monitor RNA stability using transcriptional inhibitors (e.g. cordycepin) under oxidative stresses such as high-light (Crisp PA *et al* 2017 Plant Cell). The current version represents modifications to study the influence of 5' UTR sequence composition on gene expression in Arabidopsis and wheat.

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Protoplasting is ideally performed using fully-expanded leaf tissue (leaves 4-8) of healthy 3 week-old *Arabidopsis* plants. Ensure the absence of any pests, including gnats and fungi, to maximise protoplast yield, viability, and transfection efficiency. Handle protoplasts gently (it is often recommended to keep on ice and in the dark as much as possible). Use chilled centrifuges with slow stopping (or brake off). Use pipette tips with cut ends or serological pipettes for larger volumes.

As a guide, 30 leaves in 30 mL enzyme solution yields ~2.5x10^6 protoplasts, from which 3 µg RNA can be isolated with a TRIzol-based extraction. For greater output: Yoo *et al* (2007) suggest ~100–150 leaves per 40–60 mL enzyme solution yielding 1x10^7 protoplasts.

Glucose **P212121 Catalog #Glucose**

D-Mannitol **Sigma Aldrich**

Vacuum system **Contributed by users**

KCl **Contributed by users**

MgCl2 **Contributed by users**

NaCl **Sigma Aldrich Catalog #53014**

BSA **Sigma Aldrich Catalog #A7906**

MES, sodium salt **Bio Basic Inc. Catalog #MB0611.SIZE.250g**

Razor blades **Fisher Scientific Catalog #12-640**

Centrifuge **Contributed by users**

Compound Microscope **Contributed by users**

BRAND® counting chamber BLAUBRAND® Neubauer improved New without clips, double ruled **Sigma Catalog #BR717805**

- Calcium chloride dihydrate (for cell culture; Sigma-Aldrich: C7902)
- PEG-4000 (Sigma-Aldrich: 81240)
- Onozyka Cellulase R-10 (Yakult)
- Onozuka Cellulase RS (Yakult)
- Macerozyme R-10 (Yakult)
- Potassium hydroxide or sodium hydroxide (Sigma-Aldrich)
- Flat-tip tweezers
- 0.45 µm PVDF filter (Merck, HVHP04700) and syringe (Terumo)
- 50 / 70 µm cell strainer (e.g. pluriSelect)
- NucleoBond® Xtra Midi Plus EF Plasmid extraction kit (**SKU:** 740422.10, NucleoBond Xtra Midi Plus)
- Trypan Blue or propidium iodide
- 50 mL Falcon tubes
- Serological pipettes (labtek, 650.050.110)
- Cell culture plates (Sarstedt, 83.3921.500)
- Microcentrifuge tubes
- Cluster tubes
- Flow cytometer (e.g. BD LSRII)

Handle razer/scalpel blades with care!

It is recommended to protoplasts plants kept in complete darkness prior to isolation in order to deplete their starch reserves, making for a cleaner extract.

Ensure that you have high quality supercoiled plasmid DNA (1 µg/µL) for transformation - Check out Plasmid DNA on Agarose Gel: The Secret of the 3 Bands (bitesizebio.com). We perform Midipreps on 200 mL of culture grown until OD_{600} > 2 (approx. 24 hrs) using the NucleoBond® Xtra Midi Plus EF (including NucleoBond® Finalizers) kit (Machery-Nagel).

Preparation 3w

1 Prepare the following solutions. Ensure all buffers are sterile (e.g. filter sterilize using 0.45 µm PVDF membrane). 1h

Stock solutions

- 100 mM MES-KOH (pH 5.7) [MW = 195.24 g/mol]
- 1 M Mannitol [MW = 182.17 g/mol]

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- 100 mM KCl [MW = 74.55 g/mol]
- 500 mM NaCl [MW = 58.44 g/mol]
- 500 mM $CaCl_2.H_2O$ [MW = 147.01 g/mol]
- 150 mM $MgCl_2$ [MW = 95.211 g/mol]

A	B	C
Component	Final [C]	Amount (per 100 mL)
100 mM MES-KOH (pH 5.7)	2 mM	2 mL
500 mM NaCl	154 mM	30.8 mL
500 mM CaCl2	125 mM	25 mL
100 mM KCl	5 mM	5 mL
dd-H2O		37.2 mL

W5 solution (per 100 mL)

A	B	C
Component	Final [C]	Amount (per 100 mL)
100 mM MES-KOH (pH 5.7)	4 mM	4 mL
1 M Mannitol	400 mM	40 mL
150 MgCl2	15 mM	10 mL
dd-H2O		46 mL

Mannitol-$MgCl_2$ solution (per 100 mL)

A	B	C
Component	Final [C]	Amount (per 100 mL)
100 mM MES-KOH (pH 5.7)	4 mM	4 mL
1 M Mannitol	500 mM	50 mL
100 mM KCl	20 mM	20 mL
dd-H2O		26 mL

WI solution (per 100 mL)

2 Grow Arabidopsis or wheat under 12-hour photoperiod at 21 °C and 50% relative humidity, with approx. 100 µmol photons m^{-2} s^{-1} light intensity on soil (Premium potting mix, Martins fertilizers) supplemented with fertiliser (1 g/kg Osmocote; Arabidopsis: 3 weeks, wheat: 1 week) or (Arabidopsis only) nutrient-rich media (half-strength Gamborg B5 or Murashige Skoog media supplemented with 1% sucrose; 1-2 weeks). Ensure plants are stress-free, especially from droughts, pathogens, and pests. 3w

3 Perform protoplast isolation as early in the day as possible to limit starch accumulation, which should result in a cleaner extract (can move plants to dark on the morning of isolation). Keep plants in the dark as much as possible during protoplast isolation (e.g. turn room lights off, cover digest in foil). 1d

Maceration 3h

4 Prepare enzyme solution. 15m

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A	B	C	D	E
Component	Arabidopsis		Wheat	
	Final [C]	Amount	Final [C]	Amount
100 mM MES-KOH (pH 5.7)	20 mM	2 mL	20 mM	2 mL
1 M Mannitol	600 mM	6 mL	600 mM	6 mL
100 mM KCl	20 mM	2 mL	10 mM	1 mL
cellulase R-10	1.5 % (w/v)	150 mg		
cellulase RS			1.5 % (w/v)	150 mg
macerozyme R-10	0.4 % (w/v)	40 mg	0.75 % (w/v)	75 mg
500 mM CaCl2	10 mM	200 uL	10 mM	200 uL
BSA	0.1 % (w/v)	10 mg	0.1 % (w/v)	10 mg
dd-water				800 uL

Cell wall digestion composition for Arabidopsis and wheat (per 10 mL).

Important steps:

- After mixing digestion enzymes with MES, mannitol, KCl, and water; warm solution to 55°C for 10 min with occasional swirling (inactivates nucleases and proteases, and aids solubilising enzyme).
- Allow to cool then supplement with $CaCl_2$ and BSA.
- Filter-sterilize digestion medium using a 0.45 µm PVDF membrane.

Prepare 40% PEG solution (need approximately 200 uL per transformation)

A	B	C
Component	Final [C]	Amount (per mL)
PEG-4000	40% (w/v)	400 mg
1 M Mannitol	200 mM	200 uL
500 mM CaCl2	100 mM	200 uL
dd-H2O		600 uL

40% PEG-calcium transfection solution

Add all components and mix until dissolved (1h). Make fresh on the day of transfection.

5 Prepare a clean cutting board, tweezers, and a fresh, sharp razor or scalpel. 1m

6 *Arabidopsis leaves* 10m

1. Excise fully-expanded Arabidopsis rosette leaves (typically, leaves 4-7 from well-watered 3 week-old plants).
2. Cut the mid-section of leaves (i.e. remove leaf apex and base) into approximately 1-mm strips (perpendicular to midvein) using a sharp razor blade (on a solid, clean cutting surface). Do not crush or grind the tissue, instead allow the blade to glide across tissue.
3. Immediately place leaf strips into enzyme solution, ensuring to dip both sides in the digestion medium (to ensure cutting edges are exposed to solution), using clean flat-tip forceps.

7 *Wheat leaves* 10m

1. Excise the primary leaf of approximately 1-week old wheat plants.
2. Peel away the epidermis of the abaxial layer to expose the mesophyll. This can be achieved by lightly scoring the abaxial side of the leaf with a sharp razor blade, then bending the leaf along that section to create a tab. Pull this tab using forceps to remove the epidermis (Arndell et al 2019 BMC Biotechnology).
3. Place tissue strips mesophyll side down in 0.6 M mannitol for 15 minutes (dark).
4. Continue to make strips until you have sufficient material (mesophyll strips from 1 leaf should yield at least 1 million protoplasts).
5. Transfer peels to enzyme solution.

8 Vacuum infiltrate for 30 minutes in a desiccator then release pressure gently and evenly. 30m

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9 Incubate in the dark at 23 °C for ≥ 3 hours with gentle rotation (~40 rpm). 4h

10 Add equal volume W5 and mix with gentle swirling for 10 seconds. The solution should turn green as protoplasts are released. 10s

11 2m

Check for protoplast release under light microscope (*Arabidopsis* mesophyll protoplasts are ~30-50 µm in diameter).

Resuspension 5m

12 Filter solution through 50 or 70 µm cell strainer into a falcon tube (or round bottom centrifuge tube). Keep solution on ice and shielded from strong, direct light. 1m

13 Centrifuge solution for 3 minutes at 80 rcf (soft ramp / brakes off). 3m

14 Remove majority of supernatant then resuspend protoplasts with gentle swirling (intact protoplasts will resuspend quickly whereas broken cells and free chlorophylls will not). 1m

15 Wash once more with equal volume W5 solution, centrifuge, and remove supernatant (resuspend protoplasts by swirling before moving on).

16 Add half-volume W5 solution and rest protoplasts on ice for ≤ 30 minutes. Protoplasts will settle faster to the bottom. 30m

17 Remove as much supernatant as possible without disturbing the pellet and add half-volume MMg solution. 5m

18 Centrifuge solution for 3 minutes at 80 rcf (soft ramp / brakes off). 15m

19 Remove supernatant then resuspend protoplast in 1-2 mL MMg solution.

20 Determine protoplast by counting cells on a haemocytometer. 5m

Load 10 µL of protoplast suspension and use a light microscope. Count the total number of protoplasts across 3-5 squares (count intact protoplasts only, which should be perfectly spherical).

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Protoplast density (mL^{-1} MMg) = (# protoplasts / # squares) x 10^4 x Dilution Factor

Also check for lysed cells, free chlorophylls, and epidermal debris, which reduces transformation efficiency.

21 

Adding cell stains prior to counting on a haemocytometer can help identify broken protoplasts:
- 0.1% (w/v) trypan blue
- 10 µg/mL propidium iodide (λEx = 535 nm; λEm = 617 nm)

22 Adjust protoplast density to 3x10^5 protoplasts mL^{-1} MMg.

23 Keep protoplasts in the dark at 23 °C (approx. room temperature) until ready to transfect (<8 hours). 30m

Transformation 5m

24 Prepare clean 2 mL microcentrifuge tubes and determine plasmids to be transformed.

25 Add 10-20 µg of plasmid DNA into all tubes (10-20 µL).

26 

Perform the following steps in quick succession:
(I) Add 30,000 - 60,000 protoplasts (100 - 200 µl of 3x10^5 protoplasts mL^{-1} MMg) and mix with gentle flicking.
(II) Add equal volume 40% PEG-calcium solution and mix with gentle flicking.

Notes:
- Minimise incubation time of protoplasts with DNA only to improve transfection efficiency (Arndell *et al* 2019).
- Continually swirl protoplast solution to prevent sedimentation and achieve equal distribution.
- Mass of plasmid DNA to protoplast number is important, make sure to optimise for each experiment. Currently, we are transfecting with a ratio of 1 µg : 3,000 protoplasts.

27 Incubate for at least 5 minutes at 23 °C (up to 15 minutes).

28 Add 2x volume of W5 solution (~840 µL) and mix by gentle inversion.

29 Centrifuge for 3 minutes at 80 rcf (soft ramp/ brakes off).

30 Discard supernatant and resuspend protoplasts in culture plate wells (e.g. Sarstedt 83.3921.500) with 1 mL (6-well), 0.5 mL (12-well), or 0.25 mL (24-well) W5 or WI solution (W5 is preferable for FACS due to lower mannitol,

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however, be sure to continually swirl protoplasts to avoid sedimentation).

31 Incubate protoplasts in the dark at 23 °C for 16-48 h.

32 5m

Supplement protoplast solution with 10 µg/mL propidium iodide (λEx = 535 nm; λEm = 617 nm) to stain for cell integrity. Mix with gentle flicking. Do this as close to measurement as possible.

33 Measure fluorescence using a flow cytometer (e.g. BD FACS Aria II, Invitrogen Attune NxT). 20m

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