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Protoplast isolation and transfection in a 96-well plate

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Abstract

This protocol describes a robust, high-throughput method for isolation and transfection of Arabidopsis leaf protoplasts.

Troubleshooting

Introduction

The method for protoplast isolation is based on the "Tape-Arabidopsis Sandwich" protocol reported by Wu et al. 2009 Plant Methods (https://doi.org/10.1186/1746-4811-5-16). The Tape Sandwich method is recommended for preparing large numbers of protoplasts due to the ease with which it releases protoplast cells from a high number of leaves. The transfection method is based on Yoo et al., (2007) "*Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis" Nature Protocols (https://doi.org/10.1038/nprot.2007.199) This protocol provides a detailed description of the isolation, handling and pipetting steps necessary to obtain healthy protoplasts and also consistent transfection results in 96 well plates.

Materials

2M NaCl

2M KCl

1M CaCl2

1M Mannitol

1M MES

10% BSA

2M MgCl2

W5 Solution
2mM MES (pH 5.7)
5mM KCl
154mM NaCl
125mM CaCl2

MMG Solution
4mM MES (pH 5.7)
15mM MgCl2
0.4M Mannitol

WI Solution
4mM MES(pH5.7)
0.5M mannitol
20mM KCL

Enzyme solution (made on the day)
20mM MES (pH 5.7)
20mM KCl
0.4 M Mannitol
1.5 % (W/V) Cellulase
0.4 % (W/V) Macerozyme
10mM CaCl2
0.1% BSA

PEG Solution (made on the day)
40% (wt/vol) PEG4000 in water
0.2 M mannitol
100 mM CaCl2

Procedure

Making solutions before the day of procedure

10% BSA
To make 50 ml of 10% BSA, add 5 g of BSA to 30-40 ml of water. Gently dissolve BSA to avoid foaming.Once BSA is dissolved completely, adjust volume to 50 ml with water and store at 4 ^{O}C or -20 ^{O}C for long term storage in single use aliquots in microcentrifuge tubes.

1M Mannitol (MW 182.17)
To make 50 ml of 1M Mannitol solution, dissolve 9.10 g of Mannitol in 40 ml of water. Heat it up to 65 ^{O}C in a water bath to dissolve Mannitol completely and then add water to 50 ml.

1M MES (MW 195.24)
To make 50 ml of 1M MES solution, mass out 9.76 g of MES and add water to 25 ml and add 20 drops of 10 M NaOH and vortex. The add water to 40 ml and vortex, then measure pH. It may need more NaOH to get it to pH 5.7. Then add water to 50 ml and filter-sterilise.

2M NaCl (MW 58.44)
To make 50 ml of 2M NaCl solution, dissolve 5.84 g of NaCl in 50 ml of water

2M KCl (MW 74.55)
To make 50 ml of 2M KCl solution, dissolve 7.45 g of KCl in 50 ml of water

1M $CaCl_2$(MW 110.98)
To make 50 ml of 1M $CaCl_2$ solution, dissolve 5.55 gm of CaCl2 in 50 ml of water

2M $MgCl_2$ ($MgCl_2.6H_2O$; MW 203.30)

To make 50 ml of 2M $MgCl_2$ solution, dissolve 20.33 gm of $MgCl_2.6H_2O$ in 50 ml of water

Making the W5 Solution (Ideally made ahead of time)
To make 200 ml of W5 solution, add reagents in the following way: Take 158.7 ml of MilliQ water and add the following; 400ul of 1M MES pH5.7, 500ul of 2M KCl, 15.4 ml of 2M NaCl and 25 ml of 1M $CaCl_2$.

To make 100 ml of W5 solution, add reagents in the following way: Take 79.35 ml of MilliQ water and add the following; 200ul of 1M MES pH5.7, 250ul of 2M KCl, 7.7 ml of 2M NaCl and 12.5 ml of 1M $CaCl_2$.

Making the MMG Solution (Ideally made ahead of time)
To make 20 ml of MMG solution, add reagents in the following way: Take 11.77 ml of MilliQ water and add the following; 80 ul of 1M MES pH 5.7, 8 ml of 1M Mannitol and 150 ul of 2M $MgCl_2$.

To make 40 ml of MMG solution, add reagents in the following way: Take 23.54 ml of MilliQ water and add the following; 160 ul of 1M MES pH 5.7, 16 ml of 1M Mannitol and 300 ul of 2M $MgCl_2$.

Making the WI Solution (Ideally made ahead of time)
To make 20 ml of WI solution, add 80 ul of MES, 10 ml of 1M Mannitol, 200 ul of 2M KCl and 9.72 ml of H_2O.

To make 50 ml of WI solution, add 200 ul of MES, 25 ml of 1M Mannitol, 500 ul of 2M KCl and 24.3 ml of H_2O.

Making the Enzyme solution on the day

Enzyme solution

To make 20 ml of Enzyme solution (enough for ~20 leaves), add following reagents:
400 ul of 1M MES pH 5.7 (preheated at 70 ^{O}C for 3 minutes - in heating block or water bath)
8 ml of 1M Mannitol
300 mg Cellulase,
80 mg Macerozyme,
200 ul of 2M KCl (can be added after heating step).
Heat the solution at 55 ^{O}C for 10-15 minutes (in drying cupboard), swirling occasionally, to degrade nucleases and proteases. Use a spoon/spatula to push any powder into the liquid. Cool it down to room temperature before adding. Swirl during the 10-15 mins.
200 ul of $CaCl_2$ and
200 ul of 10% BSA.

Adjust the final volume to 20 ml with water.
It is preferred to filter sterilise the enzyme solution using a 0.45 micron filter but not essential

Alt: To make 40 ml of Enzyme solution (enough for ~30-40 leaves), add following reagents:
800 ul of 1M MES pH 5.7 (preheated at 70 ^{O}C for 3 minutes - in heating block or water bath, in 1.5 ml tube)
16 ml of 1M Mannitol
600 mg Cellulase,
160 mg Macerozyme,
400 ul of 2M KCl (can be added after heating step).
Heat the solution at 55 ^{O}C for 10 minutes (in drying cupboard), swirling occasionally, to degrade nucleases and proteases. Use a spoon/spatula to push any powder into the liquid. Cool it down to room temperature before adding. Swirl during the 10 mins.
400 ul of $CaCl_2$ and
400 ul of 10% BSA.
Adjust the final volume to 40 ml with water.
It is preferred to filter sterilise the enzyme solution using a 0.45 micron filter but not essential

Isolating Protoplasts

Cut leaves from plants at the base of the leaf. This should be from from 3-5 weeks old plants - true leaf number 5-8 ideally. Number of leaves depends on the amount of transformations you want to try. 20 leaves should work for fine for a couple of 96 well plates with ~24 rnx per plate. But 30 or more leaves should be used when you have more plates

Place leaves adaxial (top side) down on coloured tape (Time tape) and then flatten the leaf so that the entire leaf is in contact with the green tape

Place Magic tape over the top of the leaf (abaxial side).

Rub the leaf to make sure that it is attached securely and sandwiched. You can use the bottom of a 15 ml conical tube for this purpose (be gentle - but ensure the midrib is in cotact with the clear Magic tape)

Then slowly peel off the magic tape to remove epidermis layer

Trim excess tape and any remaining epidermis parts of leaf can be cut out. Place exposed leaves showing their mesophyll cells into enzyme solution in petri dish. Agitate leaves in Enzyme solution for 2 hours at 200 rpm on small orbital shaker at room temp.

To make our orbital shaker start, press and hold down on the one button at the front.
Now would be a good time to make the PEG solution (see below), otherwise it can be made after you have counted the protoplasts. The protoplasts can be left in MMG solution.

Transfer the solution to a falcon tube (strain through 70 um strainer). If working with 20 ml of enzyme solution, add equal volume of W5 solution at this stage. If working with 40 ml of enzyme solution, split this equally across two tubes and to each add equal volume of W5 solution.

You can use a long (10-25 ml) pipette to transfer the protoplasts to the mesh - this is prefered if using a round Petri dish as it will lead to less spillage. When using a long pipette on protoplasts, please be gentle (turn the speed down on electric pipettes).

Centrifuge at 100g for 3 minutes (room temperature) using an Eppendorf 5430R centrifuge. The centrifuge MUST be set to Soft Spin in the menu options in order to keep the protoplasts intact.

If you do not have a soft spin option, please use a slow brake option to avoid damaging the protoplasts. We use a fixed rotor centrifuge but swinging bucket centrifuges also work well.
Remove the supernatant as much as possible without disturbing the pellet, and wash the pellet with 20 ml of W5 solution.

This would be a good step to combine the protoplasts if split because you started out with 40 ml of enzyme solution.
Centrifuge at 100g for 3 minutes (room temperature). The centrifuge MUST be set to Soft Spin in the menu options in order to keep the protoplasts intact.

If you do not have the soft spin option, please turn off the breaks to avoid damaging the protoplasts.
Remove the W5 solution - leave about 2.5 ml in the tube and then use a P1000 to remove as much as possible without disturbing the pellet.

Often about 2 ml of W5 is left in the tube before MMG is added.
Resuspend the protoplasts in 5 ml MMG solution.

Count protoplasts by loading 6 ul onto a haemocytometer (each side) - adjust concentration to 2×10^5 protoplasts/ml with MMG solution if need be. It is advised to cut the very tip of the pipette tip off (the small point of the tip can cause damage to protoplasts and can cause an uneven distrabution on the haemocytometer.

Make the PEG Solution

To make 10 ml of 40% solution, add 4 g of PEG4000 to 2 ml of water. This needs to be heated up in water bath at 60 ^{o}C for 20 minutes to dissolve PEG completely (heat in water bath for 10 minutes, then vortex and then heat again for 10 minutes), cool it down to room temperature before adding the following
2 ml of 1M mannitol and
1 ml of 1M $CaCl_2$.
Final volume is adjusted to 10 ml with water followed by vortexing.
You can calculate the amount of PEG needed as 55 ul x number of samples + 1 - 2 ml.

Transfecting protoplasts in 96 well plates

Plan out how to arrange your samples into wells. We suggest using the centre most wells to avoid evaporation from the edge of the plate and also to randomize the samples so that different samples/treatments are spread across the plate and not grouped together, potentially introducing position effects into the results. We recommend 4 replicates per plasmid being transformed into protoplasts.

Add 5 ug of plasmid DNA (1 ug/ul) to each well of the 96 well plate.

Add 10,000 protoplasts (50 ul of protoplasts adjusted to 2 ×10^5) to each well.

This step should NOT be performed using a multi-channel pipette. As protoplasts settle down quickly (due to gravity), therefore the tube containing protoplasts should be swirled continuously (but slowly) while adding protoplasts to each well. The Tube containing protoplasts is usually swirled after each 3-4 wells.

Mix the DNA and protoplasts using a multi-channel pipette 5 times.
It is recommeded to do mixing column wise and straight after mixing the DNA and protoplasts add the 40% PEG as explained in the next step
Mixing should be gentle.
It is recommended to reach the corners of the well and not only focussed on the centre of the well as protoplasts usually stay at the corner.
Avoid mixing DNA and protoplasts for the whole plate in one go.
Mixing the protoplasts and DNA into suspension immediately before adding the PEG avoids clumping and bursting of the protoplast cells.

Add 55 ul of the 40% PEG solution to each well using a multi-channel pipette (column wise) followed by mixing 10 times immediately afterwards.
Mixing of the protoplasts and DNA 5 times and then mixing with PEG 10 times take approx 1.5 minutes in one column (it is best to time this yourself). Continue with the same strategy for the rest of the columns.

Reminder: Mixing of the protoplasts and DNA immediately prior to PEG addition is mandatory as protoplasts settle down and addition of PEG to settled protoplasts leads to clumps resulting in poor transfections.

After 15 minutes of incubation, add 200 ul of W5 solution using multi-channel pipette followed by mixing 5 times to stop transfections.
As the time interval between the transfections of two columns is 1.5 minutes (or your recorded time) , therefore W5 solution should be added to next columns with an interval of 1.5 minutes (or your recorded time).

Transfections can be done successfully with a 5-15minute transfection time before stopping with W5. Choose a time that is consistent for all your experiments. 15 minutes works well when you have lots of samples to transfect in a plate, which is why we choose 15 minutes as the transfection time.

Leave the transfected protoplasts for 40-60 minutes at room temperature to settle down followed by the removal of 250 ul of supernatant using a multichannel pipette (2 × 125 ul).

Ensure that all protoplasts have settled to the bottom of the well, so that none are lost when you remove the supernatant. Placing the pipette at the bottom centre of each well during the aspirations will reduce the number of protoplasts that are lost during aspiration of the supernatant.

Resuspend the transfected protoplasts in 200 ul of WI solution using a multichannel pipette followed by gentle mixing 5 times.

Incubate the transfected protoplasts at room temperature (20 - 25 ^{O}C) for desired period of time (16 to 48 hours in our case) in continuous light or dark (light in our case), depending on the nature of experiments.