PLANT PROTOPLAST FUSION

PROTOCOL NO. PL-001

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1. Cell Type

General plant protoplast

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2. Manipulator & Electrode

Manipulator: LF101 Electro Cell Fusion Generator

Electrode(Chamber):

CUY501G4 Ms Gold Block Electrode
4mm gap, 160µl

CUY498-4 MS Stand Model Chamber Electrode
4mm gap, 2.0 ml

CUY498-10 MS Stand Model Chamber Electrode
10mm gap, 4.0 ml

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3. Pratoplast Preparation

Isolation:

Digest with a solution of 1% (w/v) Macerase, 2% [w/v] Cellulysin in 50 mM Mes-NaOH buffer (pH 5.7) with 0.6M Mannitol

Washing Procedure:

Centrifuge100 x g for 3 min
Resuspend pellet in 0.6 M mannitol

Final Protoplast Density:

1.5 - 3 x 10^5 protoplasts / ml

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4. Electroporation Setting

NOTE:

Use the following ranges and MS electrode as guidelines for establishing optimal fusion settings. These will vary depending on your particular protoplast preparation and species Once values are established under the microscope, calculate new settings and repeat the procedure with MS Stand Chamber Electrode.

Set AC Alignment Voltage :	16 - 35V
Desired Field Strength :	40 - 88 V/cm
Set AC Frequency :	1 MHz
Set Pulse Duration :	10-20 sec
Set DC Pulse Voltage :	200 - 900 V
Desired Field Strength :	0.5 - 2.25 kV/cm
Set Pulse Length :	50 µsec
Set Number of Pulses :	1
Electrode type :	4 mm gap (CUY501G4)

5. DC Pulse (Electroporation) Procedure

Protoplast Volume:

80 µl (1.5 -3 x 10^5 protoplasts / ml)

Temperature: Room temperature

MS Electrode Attachment:

1) Sterilize the MS Electrode with ethanol and dry
2) Place MS electrode on microscope stage
3) Attach C117 cables (H.S cables) to posts and tape cable to st
   to stabilize
4) Focus
5) Carefully pipette suspension between MS electrode blocks

Pulse:

Press Start switch to activate pulse sequence, alignment will occ
followed by an electroporation pulse.

Post Pulse Handling:

Observe rounding-off of hybrids through microscope.
The 2 distinct cells will form a figure-8 shape,

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6. Result

Observe microscopically for binucleate fusion hybrids

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7. Reference

Saunders JA and Bates GW, *Guide to Electroporation and Electrofusion,* Academic Press, 477 - 479, 1991

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8. Notes

1. Low ionic strength medium is recommended to obtain proper protoplast alignment in the AC field.
2. Damaged protoplasts may not align properly due to ionic leakage from the cells.
3. The electrical parameters used should be determined for each type of protoplast to be fused. If pulse-induced lysis is a problem, 1) reduce DC voltage, or 2) use shorter DC pulse lengths. Reduction of voltage will reduce lysis but will also reduce yields.
4. Reapplication of a short duration, low field strength AC field immediately after the DC pulse has been applied gives the protoplasts time to stabilize before removing from the chamber.
5. Higher densities of protoplasts will result in multi-nucleate fusion bodies, which will reduce the overall fusion yield (generally, multi-nucleate cells do not survive cell culture).

PLANT PROTOPLAST FUSION

PROTOCOL NO. PL-002

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1. Cell Type

Celery Apium graveolens L. Fusion

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2. Manipulator & Electrode

Manipulator: LF101 Electro Cell Fusion Generator

Electrode(Chamber):

CUY501G4 Ms Gold Block Electrode
4mm gap, 160µl

CUY498-4 MS Stand Model Chamber Electrode
4mm gap, 2.0 ml

CUY498-10 MS Stand Model Chamber Electrode
10mm gap, 4.0 ml

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3. Pratoplast Preparation

Callus generation: For callus generation, use 1 cm sections of petioles from seedlings at the 4-6 leaf stage. Maintain calli at 20° C in MS salts under a 12 hr dark cycle. Select fast growing, friable calli at 37 days after initiation and subculture monthly.

Celery Culture: Prepare celery suspension cultures by inoculating 50 ml of MS medium supplemented with 30 g/L sucrose, 0.25 mg/L 2,4-D., and 0.5 mg/L kinetin, with approximately 5 g friable undifferentiated callus. Grow cultures at 20° C under a 12 hr dark cycle with shaking at 100 rpm. Select fast growing, finely dispersed clones and subculture weekly by inoculating 10 ml of a 7 day old culture into 50 ml fresh medium.

Protoplast isolation: Isolate protoplasts from 3 day old suspension cultures (late lag phase) by incubation in 0.6 M mannitol, 0.1% Pectolyase Y23, and 2% Cellulysin, overnight at 30° C, with shaking at 40 rpm. After harvest, resuspend protoplastsin 0.6 M mannitol plus 0.5 mM $CaCl_2.2H_2O$.

Final Protoplast Density:

2×10^5 protoplasts / ml

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4. Electroporation Setting

Set AC Alignment Voltage :	35V
Desired Field Strength :	88 V/cm
Set AC Frequency :	1 MHz
Set Pulse Duration :	20 sec

Set DC Pulse Voltage :	400 - 800 V
Desired Field Strength :	1.0 - 2.0 kV/cm
Set Pulse Length :	99 µsec
Set Number of Pulses :	2
Electrode type :	4 mm gap (CUY501G4)

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5. DC Pulse (Electroporation) Procedure

Protoplast Volume: 20µl (2 x 10^5 protoplasts / ml)

Temperature: Room temperature

MS Electrode Attachment:

1) Sterilize the MS Electrode with ethanol and dry
2) Place MS electrode on microscope stage
3) Attach C117 cables (H.S cables) to posts and tape cable to st
   to stabilize
4) Focus
5) Carefully pipette suspension between MS electrode blocks

Pulse:

Press Start switch to activate pulse sequence, alignment will occ
followed by an electroporation pulse.

Post Pulse Handling:

Incubate in 0.6 M mannitol with gentle shaking at 20° C

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6. Result: Protoplast pearl chain formation during alignment.
Fusion frequency of 0.832 ± 0.06

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7. Reference: Lynch et al, Electrofusion of protoplasts from celery (Apium graveolens L.) with protoplasts from the filamentous *fungus Aspergillus nidulans* , Planta,V178,207-214, 1989.

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8. Notes

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2.Damaged protoplasts may not align properly due to ionic leakage from the cells.

3.The electrical parameters used should be determined for each type of protoplast to be fused. If pulse-induced lysis is a problem, 1) reduce DC voltage, or 2) use shorter DC pulse lengths. Reduction of voltage will reduce lysis but will also reduce yields.

4.Reapplication of a short duration, low field strength AC field immediately after the DC pulse has been applied gives the protoplasts time to stabilize before removing from the chamber.

5.Higher densities of protoplasts will result in multi-nucleate fusion bodies, which will reduce the overall fusion yield (generally, multi-nucleate cells do not survive cell culture).