Protocol

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Electrotransformation of *Agrobacterium tumefaciens*

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Introduction

Agrobacterium tumefaciens is a gram-negative bacteria and plant pathogen that typically causes tumor-like growths on infected plants. These tumors are prompted by the movement of transfer DNA (T-DNA) from the plasmid to the genome of the host eukaryote (often fungi). This horizontal gene transfer system, called *Agrobacterium tumefaciens*-mediated transformation or *At*MT, has become a useful technique for the insertion of modified DNA into cells to create genetically modified plant lines. The initial introduction of plasmid into *Agrobacterium* can be achieved through various techniques, including electroporation, which, compared to other techniques, has a lower risk of contamination and requires less incubation time and fewer steps. This protocol outlines the use of electroporation in the modification of *Agrobacterium* (*Agro*) with specific plasmids to later use in the generation of modified plants.

Materials

- Disarmed *A. tumefaciens* Strain (such as LBA4404)
- Binary vector (such as pBI121)

For YM Broth (1 L of broth):

- Add 0.4 g yeast extract
- 10.0 g mannitol (GoldBio Catalog # M-060)
- 0.1 g NaCl
- 0.2 g $MgSO_4$ ($7H_2O$)
- 0.5 g K_2HPO_4 ($3H_2O$)

For YM Medium Plates:

- 15 g agar per 1 L of YM broth.

For YM Storage Broth:

- Autoclaved 10% Glycerol
- 0.2 µm filter

For 1X TE Buffer:

- 10mM Tris-HCl (GoldBio Catalog # T-095)
- 1mM EDTA (GoldBio Catalog # E-210) at pH 8.0

Method

Solution Preparation

1. For 1 L of YM Broth, mix yeast extract, mannitol, NaCl, $MgSO_4$, and K_2HPO_4, adjust to pH 7.0, and autoclave.

2. For YM medium plates, prepare YM broth and add to 15 g agar and autoclave before pouring.

3. For YM Storage Broth, add 40 ml glycerol to 60 ml of YM broth, mix well, and filter-sterilize through 0.2 µm filter.

4. For 1X TE Buffer, mix 1 ml 1M Tris-HCl and 0.2 ml 0.5M EDTA, add distilled water to 100 ml, adjust pH to 8 with HCl, autoclave, and store at 4°C.

Preparation of Master Cultures

1. Streak *Agro* cells from a plate or a stab onto a YM plate and incubate at 30°C for 36-48 hours to allow to grow.

2. Use a well-isolated colony to inoculate 50 ml of YM broth in a 500 ml flask and grow cells with vigorous aeration at 30°C until OD_{550} = 0.2.

3. Transfer 5 ml of cultured cells into a chilled 15 ml falcon tube containing 5 ml of YM storage medium, mix well, and chill on ice for 10 minutes.

4. Transfer 0.5 ml of mixed cells from step 3 into an ice-chilled 1 ml Nalgene cryotube and quickly freeze in a dry-ice/alcohol bath for 10 minutes. This is the master culture.

5. Store these master cultures in a -80°C freezer.

Preparation of Electrocompetent Cells

1. Streak *Agro* cells from a master culture onto a YM plate and incubate at 30°C for 36-48 hours to allow to grow.

2. Use a well-separated colony to inoculate 50 ml of YM broth in a 500 ml flask and grow cells with vigorous aeration at 30°C until OD_{550} = 0.2.

3. Inoculate 100 µl of the cultured cells into 1.5 L of YM broth in a 2.8 L flask and incubate the cells with vigorous aeration at 30°C until OD_{550} = 1.0

Note: In general, *Agro* cells grow well in a rich medium such as LB broth. However, they are very mucoid and aggregate as cell density increases to 10^8 cells/ml. If cells are aggregated when harvested, it becomes difficult to obtain transformation efficiency for preparation. Growth in YM broth eliminates aggregation at high cell densities and allows for electrocompetent cells to be obtained consistently.

4. To harvest, transfer the cells into 500 ml centrifuge bottles and centrifuge in a cold rotor at 2600 g for 10 minutes.

5. Discard the supernatant and wash the cell pellets by resuspending cells in 500 ml of ice-cold 10% glycerol.

6. Centrifuge the cells at 2600 g for 10 minutes, then carefully pour off the supernatant. Repeat steps 5 and 6 once.

> **Note: The conductivity of the electroporation medium is a critical factor. Although many media have been used for *E. coli* and *Agrobacterium,* we have found that 10% glycerol is an effective electroporation medium and a convenient cryoprotectant for the long-term storage of frozen electrocompetent cells.**

7. Resuspend the cell pellet in ice-cold 10% glycerol to a final volume of 1.5 ml. Usually, cells can be resuspended in the 10% glycerol that remains in the centrifuge bottle. The final cell concentration should be 5×10^{10} cells/ml.

> **Note: The concentration of cells is one of the most critical factors in obtaining a high transformation efficiency of *Agro*. To obtain a transformation efficiency > 5×10^6 transformants/µg DNA it is important to have a concentration ≥ 5.0×10^{10} cells/ml. Cells in log phase yield a higher electrocompetence than cells in stationary phase. However, it is impractical to prepare large amounts of electrocompetent cells at a concentration of 5.0×10^{10} cells/ml from log phase of cultures.**

8. Cells can be used immediately or can be frozen in a dry ice-ethanol bath and stored at -80°C for 6 months.

Electroporation

1. Thaw the frozen *Agro* electrocompetent cells on ice immediately before use. If cells have been freshly prepared, keep them on ice before use.

2. Add 1.0 ml of YM broth to 15 ml Falcon tubes at room temperature.

3. Add 20-40 µl of the electrocompetent cells and 1-5 µl of DNA in TE buffer to an ice-cold 1.5 ml tube. Gently mix the samples by tapping the tube several times.

4. Transfer the samples to a microelectroporation chamber that is cooled to 4°C.

Note: Use a pulse generator with exponential decay wave form and a 0.1–0.15 cm microelectroporation chamber to generate a field strength > 15.0 kV/cm for efficient transformation. As with *E. coli,* low temperature has been found to improve the transformation efficiency of *Agro.* Thawing the frozen cells at room temperature instead of on ice decreases transformation efficiency.

5. Pulse with 16.7 kv/cm or higher with a 6-ms time constant.

Note: Because *Agro* cells are small, a high field strength is required to achieve a high transformation efficiency. As field strength increases so does transformation efficiency. Consistent transformation efficiency > 5 × 10^6 transformants/µg was achieved at a field strength of 16.7 kV/cm.

6. Transfer the cells to the YM broth in a 15 ml Falcon tube. Resuspend remaining cells in the microchamber with 200 µl of YM broth using a sterilized Pasteur pipette to remove as many cells as possible (See Table 1).

Note: The optimal broth for expression of *Agro* has been investigated. YM broth, a widely used medium with soil-borne bacteria such as *Rhizobium,* has been shown to be the best expression medium for *Agro*. The difference in expression between YM broth and LB broth can be as great as 40-fold. Moreover, the use of YM broth for *Agro* eliminates the cell aggregation that is commonly observed when LB broth is used.

Table 1. Effect of Expression Medium on the Transformation Efficiency of *Agrobacterium tumefaciens* LBA4404 Using Electroporation.

	Transformation efficiency[b]	
Expression medium[a]	YM plate	LB plate
YM broth	10	6.3
SOC broth	7.1	3.9
LB broth	0.96	0.25
EMC broth	5.3	3.2
M9 broth	0.36	0.38

[a]Medium components. LB broth: 1% bactotryptone, 0.5% yeast extract, 10mM NaCl, pH 7.0. SOC broth: 2% bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM ($MgCl_2 \cdot 6H_2O$ + $MgSO \cdot 7H_2O$), 20mM glucose, pH 7.0. EMC broth 2% bactotryptone, 1% yeast extract, 10mM NaCl, 2.5mM KCl, 1.5% succinic acid, pH 7.0. M9 broth: 1mM $MgSO_4 \cdot 7H_2O$, 1.9mM NH_4Cl, 4.2mM NaH_2PO_4, 2.5mM

Na_2HPO_4, 0.4% glucose. YM broth: 0.04% yeast extract, 1% mannitol, 1.7mM NaCl, 0.8mM $MgSO_4 \cdot 7H_2O$, 2.2mM K2HPO4·3 H_2O, pH 7.0.
Transformation efficiency (CFU/µgx10^6) was determined by pulsing 1 ng of pBI121 plasmid DNA into 20 µl of electrocompetent cells in a microelectroporation chamber at a field strength of 16.7 Kv/cm. Ten µl of electroporated mixture were diluted to 1.0 ml using different expression media, culture at 30°C for 3 hours, and plated on either YM or LB plates containing 1.5% agar with 50 µg/ml kanamycin and 100 µg/ml streptomycin. The plates were incubated at 30°C for 2 days.

7. Incubate the cells at 30°C for 3 hours while shaking at 7 g (see Table 2).

Note: *Agro* cells require an incubation period for recovery and for the expression of antibiotic resistance genes after electroporation. In *A. tumefaciens* LBA4404, it has been shown that a 3-hour expression period after electroporation results in a two-fold increase in transformation efficiency compared to a 1-hour expression period, usually done with *E. coli*.

8. Plate 100 µl onto a selective YM plate. For unknown strains and freshly prepared electrocompetent cells, plate the cells on both selective and nonselective media using serial dilutions to determine percentage of the viable cells after electroporation and the transformation efficiency.

Table 2. Effect of Expression Period on Transformation Efficiency.

	Transformation efficiency, CFU/µg[a]		
Amount of pBI121	1 hour expression	2 hour expression	3 hour expression
100 pg	16 X 10^7	2.4 X 10^7	3.3 X 10^7
1 ng	18 X 10^7	2.9 X 10^7	3.4 X 10^7
100 ng	17 X 10^7	2.9 X 10^7	3.8 X 10^7
	Total number of viable cells/reaction, CFU/mL[b]		
Amount of pBI121	1 hour expression	2 hour expression	3 hour expression
100 ng	12 X 10^9	1.3 X 10^9	1.5 X 10^9

[a]Transformation efficiency was determined by electroporating various amounts of pBI121 into 20 µl of electrocompetent cells of *A. tumefaciens* LBA4404 at a field strength of 16.7 Kv/cm, transferring 10µl of mixture into 1 ml YM broth in a 15 ml Falcon tube, and incubating at 30°C centrifuging at 7 g for different expression periods. Cells were plated onto YM medium with 50 µg/ml kanamycin and 100 µg/ml streptomycin, and the plates were incubated at 30°C for 2 days before counting the colonies. The results are the average number of three experiments and two duplicates for each experiment.
[b]Total number of viable cells were obtained as above except that cells were plated onto LB plates with 100 µg/ml streptomycin only.

Associated Products

- Mannitol (GoldBio Catalog # M-060)
- Tris-HCl (GoldBio Catalog # T-095)
- EDTA (GoldBio Catalog # E-210)

References

Lin, J. J. (1995). Electrotransformation of *Agrobacterium*. *Electroporation Protocols for Microorganisms*, 171-178.

Lurquin, P. F. (1997). Gene transfer by electroporation. *Molecular Biotechnology*, 7(1), 5-35. Doi:10.1007/bf02821542.