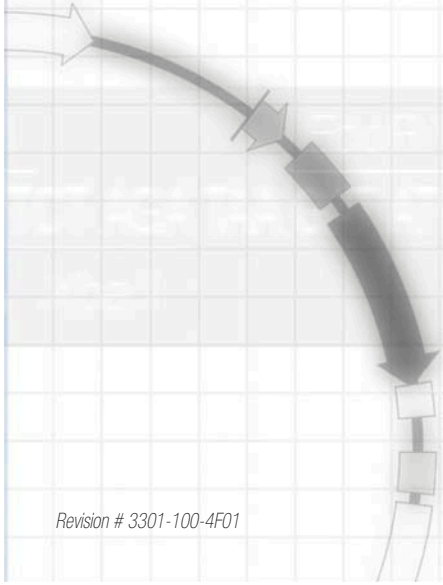
Qbiogene

Application Manual

Agrobacterium Transformation Kit

Freeze/Thaw Transformation or Electroporation of Plasmid DNA into Agrobacterium with a Single Solution



Revision # 3301-100-4F01

Q·BIOgene

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Catalog # 3301-100

25 preps

Storage:

Ambient temperature (15-30ºC)

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1. Introduction

Phytopathogenic *Agrobacterium* species are important tools for the study of plant genomics and proteomics. Native to soil, these organisms contain DNA sequences that confer virulence via the transfer of genes from the *Agrobacterium* to the plant cell. Researchers can take advantage of this ability by inserting a gene of interest into a plasmid that is then transformed into an *Agrobacterium* containing a second plasmid with virulence genes (a binary vector system)[1]. Once exposed to susceptible plant tissue, molecular signals lead to the transfer of the gene of interest into the plant cell.

Agrobacteria are difficult to work with in the lab, so preliminary subcloning and clone confirmation usually takes place in *E. coli*. Recombinant plasmid DNA is then purified from *E. coli*, and the reagents in the *Agrobacterium* Transformation Kit are used to transform DNA into the *Agrobacterium* in three basic steps. *Agrobacteria* are first grown in liquid medium, next they are transformed using either a freeze/thaw method or via electroporation. Lastly, transformed cells are plated onto selective agar plates and allowed to grow for 2-3 days into healthy colonies.

The Agrobacterium Transformation Kit contains a single transformation solution for use in either a freeze/thaw or electroporation method. Optimized Agrobacterium Medium and Agar are also included, along with Roll & Grow® Plating Beads for easy spreading of transformed cells. Once media preparation is complete, this easy transformation procedure takes less than 45 minutes with minimal hands-on time.

2. Kit Components and User Supplied Materials

2.1 Agrobacterium Transformation Kit Components

Agrobacterium Transformation Solution	2.5 ml
Agrobacterium Medium Pouch (16.5 g)	1 each
Agrobacterium Medium Agar Pouch (31.5 g)	1 each
Roll & Grow® Plating Beads	1 each
User Manual	1 each
MSDS	1 each
Certificate of Analysis	1 each

2.2 User Supplied Materials

Purified plasmid DNA from *E. coli* culture (500 ng per transformation: See section 3.5)
Agrobacterium strains (*rhizogenes or tumefaciens*)
50 ml culture tubes with caps for growth of *Agrobacterium*
Shaking incubator set to 28-30°C
Antibiotics or other selection reagents
Autoclave
Centrifuge capable of handling > 5 ml of culture at 3,500 x g

For Electroporation Only:

Electroporator
1 mm cuvettes
15 ml conical tubes (2 per transformation)

For Freeze/Thaw Transformation Only:

15 ml conical tubes (1 per transformation)
Water bath or heat block set to 30°C
Ice bucket
Liquid nitrogen, or an ethanol/dry ice slurry (deep enough to submerge > 1ml of reagents)

3. Important Considerations before Use

3.1 Electroporation vs. Freeze/Thaw Transformation

The Agrobacterium Transformation Kit provides the protocols and reagents for either a freeze/thaw or an electroporation transformation. Electroporation is the most efficient process, while freeze/thaw transformation is a shorter protocol that does not require specialized equipment. Depending on the downstream application, the freeze/thaw protocol may give an adequate number of colonies, in less time, using only one tube. Electroporation is recommended for high efficiency applications such as the construction of genomic or cDNA libraries in *Agrobacterium* and for the isolation of genes by complementation of plant mutants[2].

3.2 Selection

The selective markers in the strain being transformed and on the plasmid being used dictate what type of selective mediums to employ. Many commercially available strains of *Agrobacteria* are antibiotic resistant, and many commercially available plasmids confer antibiotic resistance or employ use of the lacZ operon to cleave β-galactosidase. Often there is a third selective marker introduced as part of the insert DNA that will be transferred into the plant (T-DNA) to confer selectability at that stage.

Antibiotics commonly used with *Agrobacterium*, and their concentration ranges, are below:

Antibiotic	Concentration
Ampicillin	20-100 µg/ml
Carbenicillin	500-1000 µg/ml
Chloramphenicol	3-25 µg/ml
Gentomycin	20 µg/ml
Kanamycin	20-100 µg/ml
Rifampin	30 µg/ml
Streptomycin	15-100 µg/ml
Sulfonamide	100 µg/ml
Tetracycline	5-10 µg/ml

3.3 Growth Medium for *Agrobacterium*

Qbiogene does not recommend the use of any growth medium other than the **Agrobacterium Medium** and **Agrobacterium Medium Agar** provided in the kit. LB Medium, as well as other versions of *Agrobacterium* media, may increase growth and processing time and will lead to few (if any) transformants.

3.4 Appropriate Experimental Controls

Negative Control: Competent bacteria without added plasmid should be plated on positive selection medium to confirm the prepared competent cells did not contain plasmid prior to use.

Positive Control: Transform a preexisting plasmid at a known concentration to confirm cell competency, check positive selection and permit calculation of transformation efficiency.

3.5 Quantity of Plasmid DNA

The amount of plasmid DNA added for each reaction plays an important role in the final transformation efficiency. Larger plasmids may need to be added at a higher quantity than smaller plasmids. If the highest possible transformation efficiency is desired, follow the electroporation protocol and optimize your specific plasmid/strain using a range of starting plasmid DNA quantities. As a starting point, Qbiogene recommends the use of 500 ng of plasmid DNA for each transformation reaction. Successful transformation has been achieved with as little as 1 ng of plasmid with both the freeze/thaw and electroporation protocols.

3.6 Choosing an Agrobacterium Strain

Many strains of *A. tumefaciens* or *A. rhizogenes* have been used to transform a variety of different plants, and the most successful strain for a particular plant should be researched prior to using this kit. While most strains in use for transformation will contain the genes necessary to move a gene of interest into a plant cell, it can be determined whether or not a particular strain of *Agrobacterium* is phytopathogenic using a simple PCR test based on the *virD2* and *ipt* genes[3].

3.7 Suitability of *Agrobacterium*-Mediated Transformation

Although *Agrobacterium* transformation remains the most common mode of transformation of dicotyledonous plants there remain many species or strains that are resistant to *Agrobacterium* infection. There has been much advancement in determining a plant's ability to be transformed, such as a polymerase chain reaction assay[4] which identifies the presence of absence of an important allele, *H2A-1*. *Agrobacterium*-mediated transformation of monocotyledonous plants such as maize and rice is also becoming routine. While the reagents in the Agrobacterium Transformation Kit should successfully allow plasmid transformation into either *A. tumefaciens* or *A. rhizogenes* species, the ultimate transformability of plants with the gene of interest is beyond the scope of this kit.

4. Safety Precautions

Some *Agrobacterium* strains are classified as plant pests, and should be only be used after obtaining all relevant state and federal permits, and after following all appropriate guidelines for use and containment. The United States Department of Agriculture (USDA) requires permits for the importation and interstate movement of plant pathogens under the authority of 7 CFR 330. Plant pathogens include nematodes, bacteria, fungi, viruses, viroids, phytoplasms, or any organisms similar to or allied with any of the foregoing, or any infectious substances which can directly or indirectly injure or cause disease or damage in any plants or parts thereof, or any processed manufactured, or other products of plants. For more information in the United States, contact the USDA (www.aphis.usda.gov/ppq/permits) or your state department of agriculture. For more information in other countries, contact your local environmental or other appropriate authority.

Agrobacterium Transformation Solution contains components that, when in contact with human tissue, may cause irritation. Wear personal protective equipment to prevent contact with the skin or mucus membranes (gloves, lab coat, and eye protection). Consult the enclosed Material Safety Data Sheet for additional details.

5. Short Protocol for Experienced Users

1. Prepare **Agrobacterium Medium** and **Agrobacterium Medium Agar** plates with the appropriate antibiotics.

2. Grow *Agrobacterium* (5 ml per transformation) to an OD_{600} of 1.0-2.0 in a 28-30˚C shaker at 200 rpm.

3. Aliquot 5 ml of culture into each sample tube.

4. Centrifuge at 3,500 x g for 5 min at room temperature or 4°C and remove supernatant.

5. Resuspend each 5 ml cell pellet in 90 µl of **Agrobacterium Transformation Solution**.

6. Add 500 ng plasmid DNA and mix gently.

7. Follow **EITHER** step 7a **OR** 7b below:

   7a. Freeze/Thaw Transformation

   1. Submerge the portion of the sample tube containing the reagents into liquid nitrogen for 1 minute.
   2. Transfer sample to a 30˚C water bath for 5 minutes.
   3. Add 0.9 ml of prepared **Agrobacterium Medium**.

   7b. Electroporation

   1. Transfer resuspended cells to a cold 1 mm cuvette.
   2. Wipe off all ice or condensation to reduce the potential for arching.

3. Electroporate the cells at a 1.5kV/cm with a pulse of 5 msec.
4. Transfer cells from the cuvette to a new 15 ml tube containing 0.9 ml of prepared **Agrobacterium Medium**.

8. Incubate samples in a 28-30˚C shaker at 200 rpm for 30 minutes to 3 hours.

9. Plate 20 µl of the recovered cells onto each selective **Agrobacterium Medium Agar** plate using **Roll & Grow® Plating Beads**.

10. Invert plates and incubate at 28-30˚C for 48-72 hours.

6. Detailed Protocol

1. Prepare **Agrobacterium Medium** with the appropriate antibiotics. Add the contents of the media pouch (16.5 g) to 1.0 L of purified water and autoclave. Allow the media to cool to 55°C before the addition of any antibiotics and bacterial inoculation. See Sections 3.2 and 3.4 for a discussion of antibiotics, selection and controls.

2. Prepare **Agrobacterium Medium Agar** with the appropriate antibiotics. Add the contents of the agar pouch (31.5 g) to 1.0 L of purified water and autoclave. Allow the media to cool to 55°C before the addition of any antibiotics, and pour into plates. See Section 3.2 and 3.4 for a discussion of antibiotics, selection and controls.

3. Inoculate prepared **Agrobacterium Medium** with a single *Agrobacterium* colony from a plate less than two weeks old. Incubate at least overnight (18-38 hours) at 28-30˚C to an OD_{600} of 1.0-2.0 in a shaker at 200 rpm. Allow 5 ml of medium for each transformation reaction. (For example, grow 25 ml of culture in order to perform 5 transformations.)

NOTE: For optimal and consistent growth, bacteria should be grown in a sealed 50 ml conical tube with 20 ml of media. Do not poke holes in the tube lid or otherwise allow aeration of the culture[5]. Do not grow cells above 32˚C as transformation efficiency can be lost. Do not overgrow the culture. If media has become visibly clear with clumps of cells, the culture should not be used for transformation. If more than 4 transformations (> 20 ml) are planned, use multiple 50 ml culture tubes. If absolute consistency is required for your experiment, pool the cell cultures together and mix gently before aliquoting into separate tubes in step 5.

4. Place **Agrobacterium Transformation Solution** on ice. If using the electroporation method, place cuvettes on ice also.

5. Aliquot 5 ml of *Agrobacterium* culture into each sample tube.

6. Centrifuge at 3,500 x g for 5 minutes at room temperature or 4°C and remove supernatant.

7. Resuspend each 5 ml cell pellet in 90 µl of ice-cold **Agrobacterium Transformation Solution**.

8. Add 500 ng plasmid DNA and mix gently.

NOTE: Some plasmids, especially large constructs, may require the use of more than 500 ng of DNA. See section 3.5 for more information.

9. Follow **EITHER** step 9a **OR** 9b below:

9a. Freeze/Thaw Transformation

1. Submerge the portion of the sample tube containing the reagents into liquid nitrogen for 1 minute.
2. Transfer sample to a 30˚C water bath for 5 minutes.
3. Add 0.9 ml of prepared **Agrobacterium Medium**.

9b. Electroporation

1. Transfer resuspended cells to a cold 1 mm cuvette.
2. Wipe off all ice or condensation to reduce the potenetial for arching.
3. Electroporate the cells at a 1.5kV/cm with a pulse of 5 msec.
4. Transfer cells from the cuvette to a new 15 ml tube containing 0.9 ml of prepared **Agrobacterium Medium**.

10. Incubate samples in a 28-30˚C shaker at 200 rpm for 30 minutes to 3 hours.

NOTE: For optimal results, transformed cells should be allowed to recover for 3 hours. In many cases, however, recovery of only 30 minutes can lead to a number of colonies that is sufficient for further growth and experimentation.

11. Plate 20 µl of the recovered cells onto selective **Agrobacterium Medium Agar** plates using **Roll & Grow® Plating Beads**:

a. Aspetically dispense 6-7 **Roll & Grow Plating Beads** onto each agar plate.
b. Replace the plate cover and gently roll the beads back and forth for approximately 30 seconds to disperse the cells. Multiple plates can be stacked and rolled simultaneously.
c. Remove the lid and roll beads from the plate into a biohazard waste receptacle.

NOTE: In most cases, plating 20 µl of the transformation reaction will result in a number of well-isolated colonies from which it is easy to calculate the transformation efficiency. If there are too many colonies, plate again using a smaller volume or dilution. If there are few colonies, plate a higher volume or spin down the 1 ml culture and remove most of the media prior to plating the entire cell pellet.

12. Replace the lid, invert the plates and incubate at 28-30˚C for 48-72 hours.

NOTE: It is normal for colonies to take several days to appear. After this initial growth period, the bacteria will return to a normal growth cycle allowing the use of an overnight media culture for downstream applications.

7. Troubleshooting

7.1 No Colonies on Agar Plate after Transformation

It can often take at least 72 hours for transformed *Agrobacterium* to form colonies on selective agar plates. Ensure that the appropriate controls are used in order to monitor growth of non-transformed *Agrobacterium* as well as those transformed with the plasmid DNA.

Ensure that the correct antibiotics and/or other selective agents were used, that they were freshly prepared, and that they were added at the correct concentrations.

Transformation may have occurred at a lower efficiency than expected. See the next section for more information.

7.2 Lower than Expected Transformation Efficiency

Transformation efficiency depends upon several critical factors, and can vary from 10^2 to 10^7 as these factors change from experiment to experiment. Most applications will not require the production of a large number of independent transformed colonies; in theory, only one colony should be used to inoculate a larger culture for the transformation of the plant itself.

In almost every case, transformation via electroporation will be more efficient than the freeze/thaw method, and may be tried if the freeze/thaw method does not yield a sufficient number of colonies.

The size of the construct being transformed can have a significant impact on the transformation efficiency. *Agrobacterium* shuttle plasmids are typically large, and the inserted DNA (T-DNA) may contain the gene of interest, its regulating sequences as well as an additional selection marker. If a low number of transformed colonies appear after a 72 hour incubation, it may be possible to increase the efficiency by repeating the transformation using more than 500 ng of plasmid DNA.

The purity and conformation of the plasmid DNA is important. Plasmid DNA should be prepared carefully so as to ensure that contaminating RNA and other proteins are absent from the preparation, and that the vast majority of recovered plasmid DNA is supercoiled. Linearized DNA will not efficiently transform bacteria.

Ensure that the *Agrobacterium* culture was grown to an OD_{600} of 1.0 to 2.0, and that 5 ml of culture is used for each transformation.

Ensure that the correct antibiotics and/or other selective agents were used, that they were freshly prepared, and that they were added at the correct concentrations.

8. Recommended Reference Format for Publication

Agrobacterium (species or strain) was transformed using the Agrobacterium Transformation Kit (Qbiogene, Inc., CA).

9. References

1. *Glick B.R., and J.E. Thompson. Methods in Plant Molecular Biology and Biotechnology, CRC Press, Inc. Boca Raton, Florida (1993).*
2. *Nickoloff J.A. Electroporation Protocols for Microorganisms, Humana Press. Totowa, New Jersey (1995).*
3. *Haas J.H., Moore L.W., Ream W., and S. Manulis. Applied and Environmental Microbiology, 2879-2884 (Aug 1995).*
4. *Zhu et al. Plant Physiology Jun; 132(2):494-505 (2003).*
5. *McCormac A.C., Elliot M.C., and D.F. Chen. Molecular Biotechnology, 9:155-159 (1998).*

10. Related Products

Cat. #	Description	Size
Plasmid Purification		
2075-400	PhoenIX™ Gigaprep Kit	5 preps
2075-300	PhoenIX™ Maxiprep Kit	25 preps
2075-350	PhoenIX™ Maxiprep Low Copy Refill Kit	12 preps
2075-600	PhoenIX™ Filter Maxiprep Kit	10 preps
2075-200	PhoenIX™ Midiprep Kit	25 preps
2075-250	PhoenIX™ Midiprep Low Copy Refill Kit	12 preps
2067-200	RapidPURE™ Plasmid Mini Kit	60 preps
2067-200	RapidPURE™ Plasmid Mini 96 Kit	96 preps
2000-200	MiniPrep Express™ Matrix	1,250 preps
Growth Media		
3301-012	Agrobacterium Medium	227 g (0.5 lb)
3301-075	Agrobacterium Medium	10 x 1 L Pouches
3301-212	Agrobacterium Medium Agar	227 g (0.5 lb)
3301-275	Agrobacterium Medium Agar	10 x 1 L Pouches
5100-035	Murashige & Skoog Basal Salts	10 L Pouch
5100-014	Murashige & Skoog Basal Salts (10X)	500 ml
5100-045	Murashige & Skoog 1/2 Strength Basal Salts (Arabidopsis Basal Salts)	10 L Pouch
5103-035	Murashige & Skoog Basal Salts with Gamborg's B5 Vitamins	10 L Pouch
5100-335	Murashige & Skoog Basal Medium (Salts, Vitamins)	10L Pouch
5100-125	Murashige & Skoog Complete Medium (Salts, Vitamins, Sucrose)	1 L Pouch
5100-114	Murashige & Skoog Complete Medium (Salts, Vitamins, Sucrose)	500 ml
5100-225	Murashige & Skoog Complete Agar (Salts, Vitamins, Sucrose, Agar)	1 L Pouch
5100-042	Murashige & Skoog Vitamins Powder	5 g

5100-044	Murashige & Skoog Vitamins (1000X)	20 ml
5101-035	Gamborg's B5 Basal Salts	10 L Pouch
5101-014	Gamborg's B5 Basal Salts (10X)	500 ml
5101-335	Gamborg's B5 Basal Medium (Salts, Vitamins)	10 L Pouch
5101-125	Gamborg's B5 Complete Medium (Salts, Vitamins, Sucrose)	1 L Pouch
5101-114	Gamborg's B5 Complete Medium (Salts, Vitamins, Sucrose)	500 ml
5101-225	Gamborg's B5 Complete Agar (Salts, Vitamins, Sucrose, Agar)	1 L Pouch
5101-042	Gamborg's Vitamins Powder	5 g
5101-044	Gamborg's Vitamins (1000X)	20 ml
5102-035	Chu's N6 Basal Salts	10 L Pouch
5102-014	Chu's N6 Basal Salts (10X)	500 ml
5102-335	Chu's N6 Basal Medium (Salts, Vitamins)	10 L Pouch
5102-125	Chu's N6 Complete Medium (Salts, Vitamins, Sucrose)	1 L Pouch
5102-114	Chu's N6 Complete Medium (Salts, Vitamins, Sucrose)	500 ml
5102-225	Chu's N6 Complete Agar (Salts, Vitamins, Sucrose, Agar)	1 L Pouch
5102-042	Chu's N6 Vitamins Powder	5 g
5102-044	Chu's N6 Vitamins (1000X)	20 ml

Plating Tools & Culture Dishes

5000-552	Roll & Grow® Plating Beads	2 tubes
5112-043	GROWTEK™ Culture Vessel	Each
5110-033	Magenta Box GA7, Pack of 8	8 boxes
5110-053	Magenta Box GA7, 5 packs of 8	Case of 40
5110-043	Magenta Box Tray (7-Way)	Each
5111-033	Petri Dishes, Sterile Polystyrene, 100 mm x 20 mm	20 plates
5111-053	Petri Dishes, Sterile Polystyrene, 100 mm x 20 mm	500 plates

Genomic DNA and RNA Purification

6540-400	FastDNA® Kit	100 preps
6560-200	FastDNA® SPIN Kit for Soil	50 preps
6045-050	FastRNA® Pro Green Kit (Plants and Animals)	50 preps
6070-050	FastRNA® Pro Soil-Direct Kit	50 preps
6075-050	FastRNA® Pro Soil-Indirect Kit	50 preps
6001-100	FastPrep® FP100A Instrument, 100V	1
6001-120	FastPrep® FP120A Instrument, 120V	1
6001-220	FastPrep® FP220A Instrument, 220V	1
1201-100	mTRAP™ Midi Kit	24 preps
1201-200	mTRAP™ Maxi Kit	6 preps
1201-300	mTRAP™ Total Kit	12 preps

11. Product Use Limitation & Warranty

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