Published in final edited form as:

Cold Spring Harb Protoc. ; 2018(1): pdb.prot094920. doi:10.1101/pdb.prot094920.

Propagating Gateway Vectors

John S. Reece-Hoyes and Albertha J.M. Walhout

Molecular Cloning collection

Abstract

Generating stocks of Entry and Destination vectors for use in the Gateway recombinatorial cloning system requires transforming them into *Escherichia coli* strain DB3.1, where they can replicate because this strain is immune to the effects of the *ccdB* gene carried in the Gateway cassette. However, mutations in the *ccdB* gene can arise at low frequency, and these mutant plasmids will consequently allow growth of standard cloning strains of *E. coli* (e.g., DH5 α). Therefore, after making new stocks of Gateway plasmids, their ability to grow in cloning strains of *E. coli* must be tested. This involves obtaining multiple stocks of vector, each arising from a single plasmid grown in a single DB3.1 bacterial colony, and transforming each stock into both DB3.1 and the preferred cloning strain of *E. coli* in a controlled fashion. Only vector stocks that effectively kill the standard cloning strain (i.e., no or few colonies are obtained after transformation) should be used in Gateway cloning reactions. The sequence can be performed in 3 d.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Agarose gels

Competent *E. coli* strain DB3.1

Competent standard cloning *E. coli* strain (e.g., DH5 α)

DNA concentration marker

Glycerol solution: 30% (v/v) in sterile water

LB medium <R> and agar plates <R>, supplemented with chloramphenicol (25 μ g/mL) and either kanamycin (50 μ g/mL) or ampicillin (50 μ g/mL)

LB medium <R> and agar plates <R>, supplemented with either kanamycin (50 μ g/mL) or ampicillin (50 μ g/mL)

Non-Gateway plasmid stock (e.g., pUC19)

TBE buffer (10×) <R>

Transformation reagents

Vector DNA (either Entry or Destination vector to be used in subsequent cloning experiments)

Equipment

Agarose gel apparatus

Culture tubes (15 mL), sterile

Glass beads, sterile

Incubator set at 37°C

Microcentrifuge

Microcentrifuge tubes (1.5 mL), sterile

Miniprep kit

Spectrophotometer capable of measuring DNA concentration (260-nm wavelength)

Water baths set at 37°C, 42°C

METHOD

1. Transform 50 pg of Gateway vector DNA into 50 µL of competent *E. coli* DB3.1 cells. Using sterile glass beads, plate the transformation mix onto LB-agar plates supplemented with both chloramphenicol (25 µg/mL) and the antibiotic prescribed by the vector of interest. Incubate the plates overnight at 37°C.

Generally, Entry vectors confer resistance to kanamycin (50 µg/mL), and Destination vectors confer resistance to ampicillin (50 µg/mL), but versions of these vectors do exist that use other antibiotics (e.g., spectinomycin).

2. Using sterile pipette tips, transfer each of the three well-separated colonies into 3 mL of LB medium supplemented with both chloramphenicol and the antibiotic prescribed by the vector of interest. Incubate the cultures overnight at 37°C with shaking.

See Troubleshooting.

3. Mix 50 µL of each overnight culture with 50 µL of 30% (v/v) glycerol solution in a sterile 1.5-mL microcentrifuge tube, and store this 15% (v/v) bacterial glycerol stock at −80°C. Use the remainder of the culture to extract the plasmid DNA using a standard miniprep procedure or kit.
4. Determine the concentration of plasmid DNA in each miniprep by using spectrophotometry or by loading 5–10 µL of the miniprep DNA on a 1% (w/v) agarose gel in 1× TBE and running it alongside DNA concentration markers. Store the plasmid DNA at −20°C.

Entry vectors are generally low-copy-number plasmids; from a 50- μ L final miniprep volume the expected yield is $\sim$ 100 ng/ μ L. Destination vectors are generally high copy number, with yields of up to 500 ng/ μ L.

5. Perform transformations.

- i. For each Gateway vector, setup two transformation reactions. Transform one 50-pg aliquot of the stock into 50 μ L of competent *E. coli* DB3.1 cells, and a second 50-pg aliquot into 50 μ L of competent cells of the standard cloning strain of choice (e.g., DH5 α).
- ii. Set up separate, parallel transformation reactions for the non-Gateway plasmid (e.g., pUC19). Transform 50 pg of the non-Gateway stock into 50 μ L of competent *E. coli* DB3.1 cells, and, in a separate reaction, into the standard cloning strain.
- iii. Using sterile glass beads, plate the transformation mixtures onto LB-agar plates containing only the antibiotic prescribed by the vector of interest.
- iv. Incubate the plates overnight at 37°C.

Because these transformations select for products of the Gateway cloning reaction, chloramphenicol should not be included in the media. Because unrecombined Gateway vectors should kill the standard cloning strain, it is necessary to verify the competence of the bacteria by including a control with a non-Gateway plasmid.

6. Assess the transformation results.

- i. Count the number of colonies that grew in each bacterial strain transformed with the non-Gateway plasmid. Calculate Q by dividing the DB3.1 colony count by the standard cloning strain colony count.
- ii. Now count the number of colonies that grew in each strain transformed with the Gateway vector. Calculate Y by first multiplying the Gateway-based DB3.1 count by Q and then dividing this product by the Gateway-based standard strain colony count.
- iii. In subsequent Gateway cloning reactions, use only the Gateway vector stocks for which Y is 1000 or greater.

The variable Q takes into account any differences in competency between the two bacterial strains. Recombination reactions using the Gateway vector stocks that pass this test should give very few, if any, colonies that contain nonrecombinant plasmid. If more Gateway vector is required in the future, this test does not have to be repeated if the plasmid is prepared using bacteria grown from the glycerol stocks made in Step 2 that match the vector stocks for which $Y > 1000$.

See Troubleshooting.

TROUBLESHOOTING

Problem (Steps 2 and 6)

There are too many colonies to count or to allow picking well-separated colonies.

Solution

Recheck the plasmid DNA concentration to ensure that the correct amount was used in the transformation. Repeat the transformation and plate smaller aliquots. Or better, plate dilutions of the final transformation mix. Check that the antibiotic is functional in the LB-agar plates by including a control to show lack of growth of a nonresistant bacterial strain that is able to grow on LB-agar plates lacking antibiotics.

Problem (Step 6)

None of the Gateway vector stocks' *Y* score is better than 1000.

Solution

Repeat the test using strains that are isogenic or as close as possible to it (see Step 1). If none of the 12 stocks pass the test, use the stock with the highest *Y* score in the required Gateway cloning reactions, and be mindful that more of the resulting colonies will need to be screened to eliminate nonrecombinant vector.

RECIPES

LB (Luria-Bertani) Liquid Medium

Reagent	Amount to add
H ₂ O	950 mL
Tryptone	10 g
NaCl	10 g
Yeast extract	5 g

Combine the reagents and shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (~0.2 mL). Adjust the final volume of the solution to 1 L with H₂O. Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle.

Media Containing Agar or Agarose

Prepare liquid media according to the recipe given. Just before autoclaving, add one of the following:

Bacto agar (for plates)	15 g/L
Bacto agar (for top agar)	7 g/L
Agarose (for plates)	15 g/L
Agarose (for top agarose)	7 g/L

Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle. When the medium is removed from the autoclave, swirl it gently to distribute the melted agar or

agarose evenly throughout the solution. *Be careful!* The fluid may be superheated and may boil over when swirled. Before adding thermolabile substances (e.g., antibiotics), allow the medium to cool to 50°C–60°C, and mix the medium by swirling to avoid producing air bubbles.

Before pouring the plates, set up a color code (e.g., two red stripes for LB-ampicillin plates; one black stripe for LB plates, etc.), and mark the edges of the plates with the appropriate colored markers. Pour plates directly from the flask; allow ~30–35 mL of medium per 90-mm plate. To remove bubbles from the medium in the plate, flame the surface of the medium with a Bunsen burner before the agar or agarose hardens. When the medium has hardened completely, invert the plates and store them at 4°C until needed.

The plates should be removed from storage 1–2 h before they are used. If the plates are fresh, they will “sweat” when incubated at 37°C. When this condensation drops on the agar/agarose surface, it allows bacterial colonies or bacteriophage plaques to spread and increases the chances of cross-contamination. This problem can be avoided by wiping off the condensation from the lids of the plates and then incubating the plates for several hours at 37°C in an inverted position before they are used. Alternatively, remove the liquid by shaking the lid with a single, quick motion. To minimize the possibility of contamination, hold the open plate in an inverted position while removing the liquid from the lid.

TBE Buffer

Prepare a 5× stock solution in 1 L of H₂O:

54 g of Tris base

27.5 g of boric acid

20 mL of 0.5 M EDTA (pH 8.0)

The 0.5× working solution is 45 mM Tris-borate/1 mM EDTA. TBE is usually made and stored as a 5× or 10× stock solution. The pH of the concentrated stock buffer should be ~8.3. Dilute the concentrated stock buffer just before use and make the gel solution and the electrophoresis buffer from the same concentrated stock solution. Some investigators prefer to use more concentrated stock solutions of TBE (10× as opposed to 5×). However, 5× stock solution is more stable because the solutes do not precipitate during storage. Passing the 5× or 10× buffer stocks through a 0.22-μm filter can prevent or delay formation of precipitates.