Published in final edited form as:

Cold Spring Harb Protoc. ; 2018(1): pdb.prot094946. doi:10.1101/pdb.prot094946.

Using Multisite LR Cloning to Generate a Destination Clone

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

Molecular Cloning collection, edited by Michael R. Green and Joseph Sambrook

Abstract

This protocol describes using the Gateway recombinatorial cloning system to simultaneously transfer a promoter and an open reading frame (ORF) from two different Entry clones into the same Destination vector using LR enzymes. A multisite cloning reaction transfers the inserts from multiple Entry clones into a single Destination vector. This type of recombination is much less efficient than transferring a single DNA fragment; however, the variety of Destination clones that can be generated in this manner is vast. In this example protocol, we describe using pDEST-MB14 to make a Destination clone that features a promoter fragment fused upstream to an ORF that is cloned in-frame with a carboxy-terminal green fluorescent protein (GFP) moiety encoded by the plasmid backbone. This method can be used as a guide for other multisite cloning reactions.

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 DH5 α bacteria (>10⁷ cfu/ μ g)

dNTP solution (1 mM stock, PCR-grade)

Gateway LR clonase II enzyme mix

GFPRV primer (5'-CTCCACTGACAGAAAATTTG-3')

GFPFW primer (5'-TTCTACTTCTTTTACTGAAGC-3')

Glycerol solutions (30%, v/v) in sterile water

LB medium <R> and agar plates <R> with ampicillin (50 μ g/mL)

ORF Entry clone in pDONR 221

pDEST-MB14 (Dupuy et al. 2007)

Stocks of nonrecombinant Gateway vectors must be prepared according to Protocol: **Propagating Gateway Vectors** (Reece-Hoyes and Walhout 2018a).

Promoter Entry clone in pDONR P4-P1R

TBE buffer (10×) <R>

TE buffer (pH 8.0) <R>

Thermostable regular DNA polymerase (e.g., *Taq*) and associated PCR buffer (10×)

Transformation reagents

Water (PCR-grade), sterile

Equipment

Agarose gel apparatus

Culture tubes (15 mL), sterile

Glass beads, sterile

Incubators set at 25°C, 37°C

Microcentrifuge

Microcentrifuge tubes (1.5 mL), sterile

Miniprep kit

PCR plates, 96 well

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

Thermal cycler

Vortex

Water baths set at 37°C, 42°C

Method

Fig. 4 in Introduction: **Gateway Recombinational Cloning** (Reece-Hoyes and Walhout 2018b) shows the multisite cloning reaction described in this protocol.

1. Combine the following reagents in a sterile 1.5-mL microcentrifuge tube on ice:

Promoter Entry clone in pDONR P4-P1R	100 ng
ORF Entry clone in pDONR 221	100 ng
pDEST-MB14	100 ng
Gateway LR clonase II enzyme mix	3 µL
TE buffer (pH 8.0)	to 15 µL

The plasmids can be quantified by spectrophotometry. Allow Gateway LR clonase II enzyme mix to thaw on ice before use. As a negative control, prepare an identical LR reaction mix with TE buffer replacing the volume of the Entry clones.

Multisite recombinations are much less efficient than single insert transfers and thus require more enzyme mix. It may be preferable to use an ORF

Entry clone that has been completely sequenced to confirm that no polymerase chain reaction (PCR) induced errors are present. Promoter Entry clones are generally not completely sequenced because errors are much less likely to have biological consequence.

Mix the reaction and incubate overnight at 25°C.

This incubation can be performed in a thermal cycler or incubator. We do not recommend incubation at regular room temperature (i.e., “on the bench”) because temperatures may vary greatly.

2. Transform each reaction mix into 150 µL of *E. coli* DH5α cells of competency >10⁷ transformants per microgram of DNA. Using sterile glass beads, plate the entire transformation mix onto LB-ampicillin plates (50 µg/mL). Incubate the plates overnight at 37°C.

Because of the low efficiency of multisite cloning reactions, the yield of colonies is highly variable but should give more than the negative control plate, which should contain no or only a few colonies (less than five). If using a different vector, a different antibiotic may be required in this subsequent and steps.

See Troubleshooting.

3. Set up amplification reactions for the colonies generated in Step 2. In sterile PCR tubes (or in the wells of a PCR plate), prepare at least 24 PCR mixes as follows.

GFPFW primer (10 µM)	1 µL
GFPRV primer (10 µM)	1 µL
dNTPs (1 mM)	2.5 µL
PCR buffer (10×)	2.5 µL
Thermostable DNA polymerase	1 unit
Water	to 25 µL

Remember to include a negative control reaction that lacks template. If a completely sequenced ORF Entry clone was used in Step 1, then fewer colonies need to be examined (up to six).

Select at least 24 bacterial colonies (from Step 2). For each colony:

- i. Use a sterile pipette tip to pick the colony.
- ii. Touch the tip to a fresh LB-ampicillin plate to propagate the colony.
- iii. Swirl the tip in a tube of PCR mix to transfer the remaining cells and discard the tip.
- iv. Incubate the LB-ampicillin plate for up to 18 h at 37°C.

The bacteria on the LB-ampicillin plates can be stored for up to 2 wk at 4°C. These primers anneal to sites in the vector either side of the promoter::ORF fusion. Diagnostic PCR using GFPRV and a promoter-

or ORF-specific forward primer may also be performed at this stage, thus removing the need to confirm insert identity by sequencing.

An alternative to bacterial PCR at this stage is to use several colonies to inoculate separate LB-ampicillin cultures and extract plasmid DNA as in Step 6, and then subject the plasmids to DNA sequencing or diagnostic restriction digests to verify insert identity.

4. Place the tubes in a thermal cycler and perform amplification using the denaturation, annealing, and polymerization times and temperatures given below.

Cycle number	Denaturation	Annealing	Polymerization
First cycle	3 min at 94°C	1 min at 56°C	1 min/kb of ORF sequence at 72°C
33 cycles	1 min at 94°C	1 min at 56°C	1 min/kb of ORF sequence at 72°C
Last cycle	1 min at 94°C	1 min at 56°C	8 min at 72°C

The conditions of the PCR may need to be optimized.

5. Load 5–10 μ L of the PCR on a 1% (w/v) agarose gel in 1 $\times$ TBE and run alongside DNA molecular mass markers. Use DNA sequencing with GFPFW and GFPRV (in separate reactions) to confirm both insert identity and functionality of the ORF–GFP junction of all the correctly sized PCR products.

When analyzing the PCR products, remember that amplification using the GFP vector primers adds 250 bp to the amplicon. It may also be preferable to sequence the promoter–ORF junction, and this may require designing other fragment-specific primers. In rare cases, it may be necessary to analyze additional colonies by PCR and/or sequencing to find a correct clone.

6. Prepare plasmid DNA from the confirmed clones:
 - i. For at least 10 clones containing the correct insert, use the bacteria from the plate generated in Step 3 to inoculate a 3-mL liquid culture of LB ampicillin (i.e., include all clones in a single culture). Grow the culture overnight at 37°C with shaking.
 - ii. In a sterile 1.5-mL microcentrifuge tube, mix 50 μ L of the overnight culture with 50 μ L of 30% (v/v) glycerol solution, and store this 15% (v/v) bacterial glycerol stock at –80°C.

Use the remainder of the culture to extract the plasmid by miniprep.

If a sequence-verified ORF Entry clone was used in Step 1, then only one correct clone need be cultured for plasmid extraction.

Troubleshooting

Problem (Step 2): The Gateway reaction fails to generate transformed bacteria.

Solution: See Troubleshooting (Steps 6 and 17) in Protocol: **Generating an Open Reading Frame (ORF) Entry Clone and Destination Clone** (Reece-Hoyes and Walhout 2018c) for the most common solutions to this problem. If the problem persists, it may be that the number of successful multisite recombination events is too low to detect using these transformation conditions. One option is to try to improve the recombination success by scaling up the reaction and transformation volumes (i.e., a 30- μ L reaction transformed into 300 μ L of cells). A second option is to use more efficient transformation methods, for example, including commercially prepared ultrahigh competent cells, or using electroporation.

Recipes

LB (Luria-Bertani) Liquid Medium

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

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.

TE Buffer, 10×

100 mM Tris-Cl (desired pH)

10 mM EDTA (pH 8.0)

Sterilize solutions by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle. Store the buffer at room temperature.

References

- Dupuy D, Bertin N, Hidalgo CA, Venkatesan K, Tu D, Lee D, Rosenberg J, Svrikapa N, Blanc A, Carnec A, et al. Genome-scale analysis of in vivo spatiotemporal promoter activity in *Caenorhabditis elegans*. *Nat Biotechnol*. 2007; 25:663–668. [PubMed: 17486083]
- Reece-Hoyes JS, Walhout AJM. Propagating gateway vectors. *Cold Spring Harb Protoc*. 2018a; doi: 10.1101/pdb.prot094920
- Reece-Hoyes JS, Walhout AJM. Gateway recombinational cloning. *Cold Spring Harb Protoc*. 2018b; doi: 10.1101/pdb.top094912
- Reece-Hoyes JS, Walhout AJM. Generating an open reading frame (ORF) entry clone and destination clone. *Cold Spring Harb Protoc*. 2018c; doi: 10.1101/pdb.prot094938