Protein Science - Combinatorial Assembly of Clone Libraries Using Site-Specific Recombination

Purpose

To generate custom expression clones using a platform that allows for many parallel cloning reactions.

Scope

This protocol includes the process of MultiSite Gateway cloning where components such as promoters, tags, and open reading frames (ORFs) are first verified as entry clones and later subcloned into a host-appropriate expression plasmid. The system is optimized for high-throughput work and produces custom expression constructs.

Before You Begin

A. This is a quick guide to MultiSite Gateway recombination cloning. Further details, including a description of how to use the various *attB* sites, can be found in Reference 1.

B. We have a variety of expression vectors and entry clones available from the following vendors:

1. https://www.kerafast.com/cat/712/dominic-esposito-phd
2. http://www.addgene.org/search/all/?q=dom+esposito

C. Additional Gateway-compatible entry clones can be found from commercial vendors, including the Human ORFeome Collaboration: https://horizondiscovery.com/en/products/gene-modulation/overexpression-reagents/orfs/pifs/ORFeome-Collaboration-Clones.

Definitions

PCR: Polymerase Chain Reaction

Materials and Equipment

A. Oligonucleotide Design

Oligonucleotides can be ordered from numerous suppliers, such as Eurofins MWG Operon (Huntsville, AL). We have found that they generally do not require high-performance liquid chromatography or gel purification, and for Gateway reactions, the amount of oligonucleotide used is so small that a 50 nmol synthesis scale is more than sufficient.

Oligonucleotides for PCR amplification should be resuspended to a concentration of 5 µM in TE (10 mM Tris-Cl [pH 8.0], 0.1 mM EDTA).

B. PCR Amplification

- 2× Phusion High-Fidelity Master Mix (New England Biolabs, Beverley, MA)
- Dimethyl sulfoxide (DMSO) (provided with the Phusion Master Mix Kit or available via commercial suppliers)
- 0.2 mL PCR Low Profile strip tubes and caps
- QIAquick PCR Purification Kit (QIAGEN, Valencia, CA)
- Ready-Load 1 Kb Plus DNA Ladder (Life Technologies, Carlsbad, CA)
- 0.8% agarose gels with TAE buffer with ethidium bromide
- Thermal cycler
- Gel documentation unit

C. BP Recombination

- BP Clonase II Kit (Life Technologies, Carlsbad, CA. Kit includes BP Clonase® II enzyme mix, 2 µg/mL Proteinase K solution.)
- QIAprep® Spin Miniprep Kit (QIAGEN, Valencia, CA)
- DH10B chemically competent cells (Life Technologies, Carlsbad, CA)
  - Store at –80°C. Do not reuse open vials.
- Superior Broth medium (AthenaES, Baltimore, MD), 40 g/L
  - Autoclave for 20 minutes.
- Lysogeny broth (LB) agar plates: LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) with 0.7% agar
  - Autoclave for 20 minutes, cool to 55°C, and add antibiotics as needed.

- Kanamycin (Sigma, St. Louis, MO), 50 µg/mL final concentration; stock solution 50 mg/mL in water, filter-sterilize, store at 4°C
- Spectinomycin (Sigma, St. Louis, MO), 100 µg/mL final concentration; stock solution 100 mg/mL in water, filter-sterilize, store at 4°C
- Falcon 2059 culture tubes (Fisher Scientific, Pittsburgh, PA)
- Supercoiled DNA ladder (Life Technologies, Carlsbad, CA)
  - Store at 4°C.
- Temperature-controlled water bath
- Tabletop centrifuge

D. LR MultiSite Recombination

- LR Clonase II enzyme mix (Life Technologies, Carlsbad, CA. Kit includes LR Clonase® II enzyme mix, 2 µg/mL Proteinase K solution.)
- Ampicillin (Sigma, St. Louis, MO), 100 µg/mL final concentration; stock solution 100 mg/mL in water, filter-sterilize, store at 4°C
- QIAprep® Spin Miniprep Kit (QIAGEN, Valencia, CA)
- BsrGI restriction enzyme (New England Biolabs, Beverley, MA)

E. Destination Vector Construction

- *E. coli ccdB* Survival-competent cells (Life Technologies, Carlsbad, CA)
  - Store at –80°C. Do not reuse open vials.
- EcoRV restriction enzyme (New England Biolabs, Beverley, MA)
- DNA Polymerase I, Klenow fragment (New England Biolabs, Beverley, MA)
- T4 DNA Polymerase/Quick Blunting Kit (New England Biolabs, Beverley, MA)
- T4 QuickLigase (New England Biolabs, Beverley, MA)
- QIAquick PCR Purification Kit (QIAGEN, Valencia, CA)
- Ready-Load 1 Kb DNA Ladder (Life Technologies, Carlsbad, CA)
- Temperature-controlled water bath
- Chloramphenicol (15 mg/mL stock in 100% ethanol)

Safety Precautions

Ethidium bromide is a mutagen and should be handled with great care. Liquid waste as well as contaminated tips and other disposables must be disposed of properly in accordance with your organization's guidelines.

Procedures

A. Oligonucleotide Design

1. Entry into Gateway cloning using BP recombination requires a PCR amplification step to add the *attB* recombination signal sequences and any other desired sequences onto the gene of interest. For clones containing protein-coding regions, proper design of the oligonucleotides for this step ensures that the correct reading frame is generated in the final Expression clone (see Note 1).
2. To clone most fragments, 18–22 bp of gene-specific 5′ and 3′ sequences are used for primer annealing. For simple Entry clones that do not contain large amounts of additional features, the recombination signal sequences given in Table 1 can be added directly to the gene-specific primers.

Table 1. Oligonucleotide Sequences for Addition of Gateway Sites

*attB*1 (to make *attL*1):	5′-GGGGACAACTTTGTACAAAAAAGTTGGC—gsp
*attB*2 (to make *attL*2):	5′-GGGGACAACTTTGTACAAGAAAGTTGG—gsp
*attB*3 (to make *attL*3):	5′-GGGGACAACTTTGTATAATAAAGTTGGC—gsp
*attB*4 (to make *attL*4):	5′-GGGGACAACTTTGTATAGAAAAGTTGGC—gsp
*attB*5 (to make *attL*5):	5′-GGGGACAACTTTGTATACAAAAGTTGGC—gsp
*attB*1rev (to make *attR*1):	5′-GGGGCCAACTTTTTTGTACAAAGTTG—gsp
*attB*2rev (to make *attR*2):	5′-GGGGCCAACTTTCTTGTACAAAGTGG—gsp
*attB*5rev (to make *attR*5):	5′-GGGGCCAACTTTTGTATACAAAGTTGA—gsp

gsp, gene-specific primer (should contain 18–22 bp of 5′ or 3′ of gene; in reverse primers this sequence must be the reverse complement of the sense strand of the gene)

Underlined sequences in the *att* sites identify the actual recombination site overlap regions that provide specificity.

3. Long PCR primers are required for more complicated tagging in Entry clones. Introduction of protease cleavage sites or epitope

tags often leads to oligonucleotide lengths in excess of 60 nt. In our experience, such long oligonucleotides often are of reduced quality, containing higher numbers of mutations or deletions. This may require more clones be sequenced in order to avoid errors. A technique known as adapter PCR can be used to avoid this problem.

4. Adapter PCR involves the use of multiple nested primers to add long 5′ or 3′ sequences to the gene of interest. First, a primer that contains the gene-specific portion and part or all of a tag sequence (such as an epitope tag) is added to the PCR. After a few rounds of amplification, a second primer, which contains the appropriate *attB* recombination signal and 12–16 nt of overlapping sequence with the first primer, is added. A mixture of PCR products will be produced, but only the full-length product will have the *attB* recombination sites necessary for recombination to occur.

5. Adapter PCR can be used on both ends simultaneously by adding two different adapter primers. It is also possible to nest multiple levels of adapter PCR to insert long 5′ or 3′ sequences if necessary. Often, the same adapter primers can be used for any gene that has a particular 5′ sequence, thus minimizing the cost and number of oligos that need to be generated for library construction.

6. In cases where multiple large genes are to be combined (e.g., a fusion of a protein of interest with a second protein of interest), or when large deletions are desired, overlap PCR can be used (see Reference 8). In this process, two separate PCR amplifications are carried out with 20–25 bp of overlapping sequence between the 3′ end of gene 1 and the 5′ end of gene 2. A third PCR is then carried out using the first 2 PCR products as templates along with flanking primers containing the *attB* sites. Again, the presence of the *attB* sites during only the last round of PCR ensures that no other side-products will be able to be cloned.

7. Oligonucleotides for sequence verification of Entry clones are required in addition to oligonucleotides for amplification. We generally order 1 primer for every 600 bp of sequence, along with an additional primer on the reverse strand that is able to sequence back through the start of the gene. Typical primer lengths are 20–24 nt, and they can be selected manually or with the assistance of many common molecular biology computer programs. Standard Gateway sequencing primers can also be used to sequence into the gene of interest from both directions in the Entry clone.

B. PCR Amplification

1. To a 200 µL thin-walled PCR tube, add 1 µL of each 5 µM oligonucleotide primer, 0.75 µL DMSO (see Note 2), 50–100 ng template DNA (see Note 3), and water to 12.5 µL final volume.

2. Add 12.5 µL 2× Phusion High-Fidelity Master Mix, mix well, and carry out the PCR amplification using the following parameters: initial denaturation at 98°C for 30 seconds, followed by 20 cycles (each 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 30 seconds per kb of the expected product), followed by a 10-minute final elongation at 72°C and cooling to 4°C (see Note 4).

3. For adapter PCR (see Procedures A.4), after five cycles of amplification, pause the thermal cycler and add 1 µL of 5 µM adapter primer(s) to the tubes. Continue cycling for an additional 20 cycles.

4. There are 5 cycles between adapter additions and at least 20 additional cycles after the final adapter is added.

5. If only a small amount of template DNA is available, increase the number of overall cycles from 20 to 30. This will increase the likelihood of PCR errors but may improve PCR product yield.

6. After cycling, load 5 µL of the PCR product on a 0.8% agarose gel to verify size by comparison to a linear DNA standard, such as the Ready-Load 1 Kb DNA Ladder (see Note 5).

7. Purify the PCR product using the QIAquick PCR Purification Kit following the manufacturer's protocol and elute the DNA in a final volume of 50 µL (see Note 6).

C. BP Recombination

1. Add the following reagents to a microcentrifuge tube in the order given (the total reaction volume should be 5 µL): 2 µL H_2O, *attB*-flanked PCR fragment (15–150 ng, see Note 7), 150 ng Donor vector (see Note 8), and 1 µL of BP Clonase II. A master mix can be used for all reagents except for BP Clonase II, which must be added last. Mix for 2 seconds by gentle vortexing.

2. Incubate the reaction mixture for at least 1 hour at 30°C.
3. Add 1 µL of 2 mg/mL Proteinase K to inactivate the BP Clonase, then incubate for 15 minutes at 37°C.
4. Add 1 µL of the BP reaction to a microcentrifuge tube containing 20 µL of chemically competent *E. coli* DH10B and incubate the tube on ice for 10–20 minutes (see Note 9).
5. Heat-shock the cells in a 42°C water bath for 45 seconds and immediately add 80 µL of LB medium. Shake the reaction for 1 hour at 37°C.
6. Spread 100 µL of the transformation mix on LB agar plates containing the proper antibiotic (see Note 10) and incubate the plates overnight at 37°C. A good BP cloning result with a standard-length (1 kb) insert should yield greater than 200 colonies per transformation.
7. Pick 2–4 separate Entry clone colonies into Falcon 2059 culture tubes containing 2 mL of SB medium with antibiotic and grow them overnight at 37°C with 200 rpm shaking.
8. Spin 1 mL of the culture in a microcentrifuge to pellet the cells, then isolate the plasmid by using the QIAprep Spin Miniprep Kit, eluting the DNA in 75 µL of elution buffer (see Note 11).
9. Verify the size of the plasmid by using agarose gel electrophoresis. Load 1 µL of the purified Entry clone DNA on a 0.8% agarose gel and compare sizes to the Supercoiled DNA ladder.
10. Verify the sequences of properly sized Entry clones to ensure that no oligonucleotide or PCR-generated errors have been introduced (see Note 12).
11. Make glycerol stocks of the *E. coli* strains containing Entry clones by adding 100 µL filter-sterilized 60% glycerol to 300 µL of culture. After mixing and incubation at room temperature for 5 minutes, these stocks can be frozen at –80°C and used to start new cultures if more Entry clone DNA is required in the future.

D. MultiSite LR Recombination

1. Add the following reagents to a microcentrifuge tube in the order given (the total reaction volume should be 10 µL): 1–5 µL H_2O, 50 mg of each Entry clone DNA (see Note 13), 100 ng Destination vector DNA (see Note 14), and 2 µL LR Clonase II (see Note 15).
2. Incubate the reaction mixture overnight at 25°C (see Note 16).
3. Add 1 µL of 2 mg/mL Proteinase K to inactivate the LR Clonase II and incubate for 15 minutes at 37°C (see Note 17).
4. Add 1 µL of the LR II reaction to a microcentrifuge tube containing 20 µL of chemically competent *E. coli* DH10B and incubate the tube on ice for 10–20 minutes (see Note 9).
5. Heat-shock the cells in a 42°C water bath for 45 seconds and immediately add 80 µL of LB medium. Shake the reaction for 1 hour at 37°C.
6. Spread 100 µL of the transformation mix onto an LB agar plate containing the correct antibiotic (often ampicillin, but check the Destination vector information) and incubate it overnight at 37°C.
7. Pick two separate colonies into Falcon 2059 culture tubes containing 2 mL of SB medium and the correct antibiotics. Grow them overnight at 37°C with 200 rpm shaking.
8. Spin 1 mL of the culture in a microcentrifuge tube to pellet the cells, and isolate the plasmid by using the QIAprep Spin Miniprep Kit, eluting the DNA in 50 µL of elution buffer (see Note 18).
9. Verify the size of the plasmid by using agarose gel electrophoresis. Load 1 µL of the purified Expression clone DNA on a 0.8% agarose gel and compare sizes to the Supercoiled DNA ladder.
10. Additional confirmation of the Expression clone should be carried out by restriction enzyme analysis (see Note 19). 1 µL of Expression clone DNA can be digested using BsrGI restriction endonuclease for 1 hour at 37°C.
11. Glycerol stocks of the *E. coli* strains containing MultiSite clones should be made by adding 100 µL filter-sterilized 60% glycerol to 300 µL of culture. After mixing and incubating at room temperature for 5 minutes, these stocks can be frozen at –80°C and used to start new cultures if more Expression clone DNA is required in the future.

E. Destination Vector Construction

1. Cassettes for generating new Destination vectors generally contain two *attR* sites flanking a set of positive and negative selection markers. In the case of standard Gateway vectors, the negative selection is done by the

ccdB gene, and the positive selection by the CAT gene for chloramphenicol resistance.

2. To generate new Destination vectors, transfer vectors containing attR4-attR2 (pSpcRFA42) or attR4-attR3 (pSpcRFA43) cassettes flanked by a pair of EcoRV restriction sites can be used. Digestion with EcoRV produces a Gateway cassette, which can be introduced into any vector that has a blunt cutting site or that has been blunt-ended.
3. Prepare the Destination vector cassette by digesting a suitable amount of the parental vector with EcoRV and purifying it using the QIAquick PCR Purification Kit. This cut material can be saved for future use, so we suggest doing a single large reaction and storing the cut material at –20°C for subsequent vector conversion work (see Note 20).
4. For production of Destination vectors using a blunt cutting site, 1 µg of the vector of interest must first be linearized using a restriction endonuclease that generates blunt ends. After digestion, 2 µL of the linearized product should be confirmed on a 0.8% agarose gel to verify size by comparison to a linear DNA standard, such as the Ready-Load 1 Kb DNA Ladder (see Note 21). Purify the linearized vector using the QIAquick PCR Purification Kit following the manufacturer's protocol, followed by a 50 µl elution.
5. Production of Destination vectors using a cohesive end cut site will require enzymatic blunting to allow correct insertion of the Gateway cassette fragment. The purified linearized vector with cohesive ends must be treated with DNA Polymerase 1, Klenow fragment (for 5′ DNA overhangs), or T4 DNA Polymerase (for 3′ DNA overhangs). Standard manufacturer's protocols (New England Biolabs, Beverly, MA) can be used for both reactions. Use QIAquick PCR Purification and subsequent 50 µL elution after the Klenow protocol. The product of the New England Biolabs Quick Blunting Reaction is used directly for Step 6.
6. Use 1 µL of the blunt-ended linearized vector in a ligation reaction with 3 µL of the digested Gateway cassette of choice. Bring the reaction to 10 µL with water, then add 10 µL of the 10× Ligase Reaction Buffer, followed by 1 µL of T4 DNA Ligase. Incubate the reaction at 25°C for 10 minutes (see Note 22). Based on concentrations of the vector and insert, these amounts may vary, with the goal of a 1:3 vector:insert ratio.
7. Add 1 µL of the ligation reaction to a microcentrifuge tube containing 20 µL of chemically competent *E. coli* ccdB Survival cells and incubate them on ice for 10–20 minutes (see Note 8).
8. Heat-shock the cells in a 42°C water bath for 45 seconds and immediately add 80 µL of LB medium. Shake the reaction for 1 hour at 37°C.
9. Spread 100 µL of the transformation mix on LB agar plates containing the proper antibiotics (including 15 µg/mL chloramphenicol for Gateway cassette selection) and incubate the plates overnight at 37°C.
10. Pick 6–8 separate Destination vector colonies into Falcon 2059 culture tubes containing 2 mL of SB medium with antibiotic and grow them overnight at 37°C with 200 rpm shaking.
11. Spin 1 mL of the culture in a microcentrifuge to pellet the cells, then isolate the plasmid by using the QIAprep Spin Miniprep Kit, eluting the DNA in 50 µL of elution buffer.
12. Verify the size of the plasmid by using agarose gel electrophoresis. Load 1 µL of the purified Destination vector DNA on a 0.8% agarose gel and compare sizes to the Supercoiled DNA ladder.
13. Carry out additional confirmation of the Destination vector colonies by restriction enzyme analysis, using an enzyme that will produce diagnostic bands to provide information on the orientation of the Gateway cassette (see Note 23). In addition, sequence confirmation across the Gateway cassette boundaries is strongly suggested (see Note 24).
14. Glycerol stocks of the *E. coli* strains containing Destination vector clones should be made by adding 100 µL filter-sterilized 60% glycerol to 300 µL of culture. After mixing and incubating at room temperature for 5 minutes, these stocks can be frozen at –80°C and used to start new cultures if more Destination vector is required in the future.

Notes

1. It is a common problem for new Gateway users to confuse the reading frames and directions of the "reverse" *att* sites needed

to make *attR* containing Entry clones. We strongly recommend the use of in silico modeling of Gateway cloning prior to ordering oligonucleotides. Several products can be used for this purpose, including Vector NTI (Life Technologies, Carlsbad, CA) and Clone Manager (SciEd Software, Cary, NC). Many other programs can also be tricked into carrying out Gateway reactions by pretending that the Gateway *att* sites are restriction sites. In any case, it is always best to validate the reactions in the computer before spending time and money on incorrect sequences.

2. The addition of 3% DMSO to the PCR can help to reduce the effects of GC-rich primers or template DNA. Often it is not required, but we have seen no detrimental effect from including it in most PCRs. If templates are very AT-rich, we suggest omitting the DMSO.

3. The use of large amounts of template DNA helps to dramatically reduce PCR errors by forcing the use of original template molecules instead of possibly error-prone PCR products for subsequent PCR cycles. If limited template is available, this amount can be reduced by 10- to 20-fold, but the chances of PCR errors will likely increase.

4. Phusion polymerase has become the standard PCR reagent in our lab due to its robust activity and extremely high fidelity. Other polymerases can also be used, but we recommend using only high-fidelity enzymes to limit the introduction of mutation(s) during the PCR procedure, particularly for generating long amplicons. The suggested conditions are optimized for use on Bio-Rad or Applied Biosystems PCR machines; some optimization may be required if PCR machines with slower thermal ramping times are used.

5. The appearance of a properly sized band on an agarose gel does not guarantee the proper addition of adapter primers during adapter PCR. In most cases, the extra length of the adapter primer does not change the overall DNA size sufficiently to detect on a gel. For this reason, if there are failures to BP clone products of an adapter PCR reaction, it is sometimes worth validating that the adapters are working by carrying out two separate PCRs instead of a single adapter PCR.

6. Column purification of PCR products is only successful for products >150 bp in length. For smaller products, a DNA precipitation with polyethylene glycol can be carried out as detailed in the Gateway product manuals. Failure to purify PCR products will often lead to a large number of primer-dimers (small fragments caused by primer misannealing), which will clone very efficiently in the BP reaction. In extreme cases, gel purification may be necessary to eliminate these products entirely.

7. Generally, the more PCR product used in the reaction, the higher the efficiency will be. Particularly with long PCR products (>5 kb), the higher end of the concentration range should be used. Be aware that with adapter PCRs, the effective concentration of PCR product with both *attB* sites may be lower than the total concentration.

8. There are numerous Donor vectors available—these are the *att*P containing vectors that become the backbone of the Entry clone after the BP reaction. pDONR221 is a common vector for use with *att*1-*att*2 clones. Other vectors are required for different *att* site combinations and can be obtained from Life Technologies or the authors. Note that Donor vectors must be propagated in *E. coli ccdB* Survival or another strain that is resistant to the CcdB toxin.

9. DH10B is a recommended *E. coli* strain for Gateway reactions. However, it can be substituted with any other *recA endA* strain (such as TOP10 or DH5α) if necessary. Strains used must not carry the F′ episome (such as in XL1-Blue), as it will interfere with the Gateway negative selection. For good results, be sure that the competent cells have a transformation efficiency of at least 1×10^8 cfu/µg. Electrocompetent cells can also be used instead of chemically competent cells; however, the only advantage would be in the case of a very low efficiency reaction (such as with a very long insert)—usually the number of colonies obtained with standard chemically competent cells is more than sufficient.

10. Most Donor vectors contain either kanamycin or spectinomycin resistance markers, but be sure to verify the correct antibiotic before use.

11. Many commercial kits are available for generating plasmid DNA from *E. coli*. A standard alkaline lysis plasmid prep will also work in most cases, but these tend to add significant time to the process.

12. Due to the presence of inverted *att* site repeats in some Entry clones, sequencing of very small inserts (<400 bp) can be difficult. For this purpose, use specially designed blocking oligos (see Reference 9) to eliminate the problem, or simply sequence from the inside of the insert using gene-specific primers.

13. In MultiSite recombination, the ratio of Entry clones to Destination vector is crucial. Addition of extra Entry clone may improve efficiency, but this will likely also increase the chance of cotransformation of Expression clone DNA and Entry clone DNA into the same cell. We suggest a maximum of 50 ng per Entry clone and 100 ng of Destination vector to avoid cotransformation problems. However, increases in efficiency, if needed, can sometimes be achieved by increasing the amount of Destination vector.

14. Destination vectors can be added as either supercoiled plasmids or plasmids that have been linearized within the Gateway cassette. Contrary to the manufacturer's assertion, using linearized Destination vector will actually improve LR reaction efficiency two- to fivefold. However, due to the high efficiency of the LR reaction, we do not generally find the extra effort required to prepare and purify linearized Destination vector to be worthwhile except in the extreme cases of 4- and 5-fragment cloning.

15. Although a specific product (LR Clonase Plus) is marketed as a requirement for MultiSite Gateway reactions, this reagent is not actually necessary for performing MultiSite LR reactions. Standard LR Clonase II will work just fine as long as the concentrations of Entry clones and Destination vectors are accurately maintained. Under proper reaction conditions, 95% of reactions will proceed equally well with the standard Clonase reaction mix.

16. Due to the complexity of the MultiSite LR reaction, overnight incubation at 25°C is required to increase recombination efficiency and will produce a more than sufficient number of colonies. Shorter incubation times do not produce enough colonies on a consistent basis to be worthwhile, particularly with higher numbers of fragments.

17. Failure to treat the LR reactions with Proteinase K will result in dramatically reduced colony counts due to the inability of the DNA to transform while coated with Clonase proteins.

18. Many commercial kits (see Note 11) are available for generating plasmid DNA from *E. coli*. The QIAprep Spin Miniprep Kit is used here, as many of the LR reaction products we use are destined for transfection into mammalian cells. This kit provides DNA of both a reasonable quality and yield and produces DNA with a relatively low level of endotoxin, which can be directly transfected into most mammalian cells.

19. Standard Gateway reactions are usually so efficient and accurate that further confirmation of Expression clones is not necessary. However, as the fragment number increases, the slight possibility of unusual recombination byproducts with MultiSite reactions exists, so restriction enzyme analysis is often worthwhile. Some of the Gateway *attB* sites can be cleaved with the restriction enzyme BsrGI, which will generally yield diagnostic bands to allow verification of insert sizes. Alternatively, other restriction sites can be employed within the genes or flanking regions depending on the particular circumstances.

20. Note that the cassette does not need to be gel-purified away from the backbone of the plasmid in many Gateway cassette transfer vectors. The pSpcRFA42 and pSpcRFA43 vectors contain a spectinomycin-resistant backbone that will not be selected for in the final transformation of these vectors, allowing the use of cut but not purified vector for the reactions.

21. It is important to ensure that all of the parental plasmid has been digested in this reaction. Any leftover transfer vector can confer chloramphenicol resistance during cotransformation and can lead to false-positive colonies.

22. The suggested ratio of vector:insert in this ligation reaction is 1:3 on a molar basis. However, we find that it is usually good enough to estimate the amounts visually on a gel rather than performing calculations. In general, addition of an excess of cassette to vector yields good results. There is a strong selection for the proper clones due to the chloramphenicol selection on the Gateway cassette and the resistance marker on the vector to be converted.

23. With blunt insertion, there is obviously a 50% chance of obtaining forward- or reverse-orientation inserts. Several unique restriction sites can be found within the Gateway cassettes at nonsymmetric locations. These include EcoRI, NotI, and BamHI. Depending on the restriction map of your vector, one of these may be useful as a diagnostic for directionality of the insert.

24. If allowed to proceed too long or in suboptimal buffer conditions, EcoRV reactions tend to chew back 1–3 nt at the cut sites. While this generally does not affect the function of a Gateway reaction (the EcoRV site is not integral to the site), it can dramatically affect the reading frame if the vector contains a protein-coding region. For this reason, we suggest sequencing with primers internal to the Gateway cassette to ensure that the boundary sequences are accurately known.

References

1. Wall, VE, et al. Combinatorial Assembly of Clone Libraries Using Site-Specific Recombination, in *DNA Cloning and Assembly Methods*, S. Valla and R. Lale, editors. 2014, Humana Press: Totowa, NJ. p. 193-208.

2. Hartley JL, Temple GF, Brasch MA (2000). DNA cloning using in vitro site-specific recombination. *Genome Res.* 10:1788–1795. PubMed, CrossRef, Google Scholar.

3. Esposito D, Garvey LA, Chakiath CS (2009). Gateway cloning for protein expression. *Methods Mol Biol.* 498:31–54. PubMed, CrossRef, Google Scholar.

4. Cheo DL, Titus SA, Byrd DR, et al. (2004). Concerted assembly and cloning of multiple DNA segments using in vitro site-specific recombination: functional analysis of multi-segment expression clones. *Genome Res.* 14:2111–2120. PubMed, CrossRef, Google Scholar.

5. Sasaki Y, Sone T, Yoshida S, et al. (2004). Evidence for high specificity and efficiency of multiple recombination signals in mixed DNA cloning by the Multisite Gateway system. *J Biotechnol.* 107:233–243. PubMed, CrossRef, Google Scholar.

6. Sasaki Y, Sone T, Yahata K, et al. (2008). Multi-gene gateway clone design for expression of multiple heterologous genes in living cells: eukaryotic clones containing two and three ORF multi-gene cassettes expressed from a single promoter. *J Biotechnol.* 136:103–112. PubMed, CrossRef, Google Scholar.

7. Sone T, Imamoto F (2012). Methods for constructing clones for protein expression in mammalian cells. *Methods Mol Biol.* 801:227–250 PubMed, CrossRef, Google Scholar.

8. Hopkins RF, Wall VE, Esposito D (2012). Optimizing transient recombinant protein expression in mammalian cells. *Methods Mol Biol.* 801:251–268. PubMed, CrossRef, Google Scholar.

9. Horton RM, Cai ZL, Ho SN, et al. (1990). Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques.* 8:528–535. PubMed, Google Scholar.

10. Esposito D, Gillette WK, Hartley JL (2003). Blocking oligonucleotides improve sequencing through inverted repeats. *Biotechniques.* 35:914–920 PubMed, Google Scholar.