Published in final edited form as:

Cold Spring Harb Protoc. ; 2018(1): pdb.top094912. doi:10.1101/pdb.top094912.

Gateway Recombinational Cloning

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

Abstract

The Gateway recombinatorial cloning system was developed for cloning multiple DNA fragments in parallel (e.g., in 96-well formats) in a standardized manner using the same enzymes. Gateway cloning is based on the highly specific integration and excision reactions of bacteriophage λ into and out of the *Escherichia coli* genome. Because the sites of recombination (“*att*” sites) are much longer (25–242 bp) than restriction sites, they are extremely unlikely to occur by chance in DNA fragments. Therefore, the same recombination enzyme can be used to robustly clone many different fragments of variable size in parallel reactions.

General Cloning Principles

Many experimental approaches use reporter constructs to aid in understanding protein expression and function. Typically, these constructs are plasmid vectors designed to express a reporter protein that indicates the spatiotemporal expression and/or the function of a protein of interest. A reporter protein may be expressed alone under the sole control of a regulatory DNA sequence of interest, or it may be joined in a translational fusion to a protein of interest. A reporter protein can be a fluorescent protein that can be visualized (e.g., the green fluorescent protein, GFP), an enzyme whose activity can be determined (e.g., luciferase), or a tag that brings a specific biochemical property to the fusion (e.g., glutathione-*S*-transferase, GST). For example, a construct in which GFP is fused to a protein of interest, having its expression controlled by the corresponding gene promoter, can show where and when the endogenous protein is expressed by observing the GFP expression pattern.

Reporter constructs are typically made by transferring the inserted sequence—an open reading frame (ORF) and/or a regulatory DNA fragment (e.g., a promoter)—into a plasmid that encodes the reporter protein, such that the ORF shares the reading frame of the reporter. Conventional cloning methods use restriction endonucleases to generate both vector backbone and insert(s) with compatible ends so that they can be joined together to form a circular plasmid, using DNA ligase. In this way, construct creation is limited by the presence or absence of appropriate digestion sites within both the DNA fragment and the vector, and it is highly unlikely that any two reporter constructs would use the same combination of restriction enzymes. These factors make generating more than a few different reporter constructs using conventional cloning a challenge and generating genome-wide collections for high-throughput research impossible.

Basic Principles and Applications of Gateway Cloning

The Gateway recombinatorial cloning system was developed for cloning multiple DNA fragments in parallel (e.g., in 96-well formats) in a standardized manner using the same enzymes (Hartley et al. 2000; Walhout et al. 2000; Reboul et al. 2001). Gateway cloning is based on the highly specific integration and excision reactions of bacteriophage λ into and out of the *Escherichia coli* genome (Hartley et al. 2000). The Gateway cloning system uses two different enzyme mixtures, each of which performs a different type of recombination reaction. The BP clonase enzyme mix recombines *attB* sites with *attP* sites, generating *attL* and *attR* sites; whereas the LR clonase enzyme mix catalyzes the reverse reaction (Fig. 1A). Each recombination event is precise, with no nucleotides either gained or lost. Recognition of *att* sites is extremely specific (i.e., BP clonase enzymes never use *attL* or *attR* sites), thus every recombination reaction generates only one set of derivatives. The four types of *att* sites differ by the presence/absence of “arms” (Fig. 1B) on either side of a core 25-bp “recognition region.” The arms contain interaction sites for the recombination enzymes. Within the recognition region is a 7-bp “asymmetric overlap” that is the site at which the DNA is cut and rejoined (Fig. 1C). Introducing nucleotide changes within the 25-bp recognition region (Fig. 1C) has been used to engineer different subtypes of *att* sites that recombine only with each other (i.e., *attB1* sites with *attP1* sites, but not with *attP2* or *attP3* sites). These different sets of *att* sites facilitate cloning of each DNA fragment in only one orientation (Fig. 2) into each vector (e.g., by placing a different *attB* site at either end).

For every DNA fragment of interest (e.g., promoter, ORF, 3' UTR), an “Entry clone” is first generated by a BP Gateway cloning reaction into a “Donor vector” (Fig. 2). Typically, the DNA fragment containing the ORF or regulatory sequence is initially amplified by PCR using long (~50 bp) tailed primers with the 3' part of the primers specific for the DNA of interest and the 5' tail of the primers containing appropriate *attB* sites. This PCR product is then transferred into a Donor vector with compatible *attP* sites. The DNA fragment in an Entry clone is now available for transfer into many types of “Destination vectors” by a Gateway LR cloning reaction (Fig. 3). Importantly, the primers used initially to clone every ORF can be designed to remove the endogenous start/stop codons and to match the reading frame used by all Destination vectors that express either amino-terminal or carboxy-terminal fusion proteins. This reading frame is unchanged by transfers between vectors because Gateway recombinations are precise. More advanced “multisite” Gateway LR reactions can be used to incorporate the DNA fragments from more than one Entry clone (e.g., promoter and ORF) into a single Destination construct (Fig. 4). This approach is extremely useful for generating complex constructs, in which, for instance, a promoter and ORF are fused together to a GFP-encoding ORF.

To select the desired recombination product, all Entry and Destination vectors contain a “Gateway cassette” as well as different antibiotic-resistance genes. The Gateway cassette contains a chloramphenicol-resistance gene and the toxic *ccdB* gene (Bernard and Couturier 1992; Miki et al. 1992) that confers lethality to standard *E. coli* strains (e.g., DH5 α). The recombination event swaps the DNA fragment of interest with the Gateway cassette, thus enabling growth of DH5 α bacteria on media containing the required antibiotic (e.g., kanamycin for Entry clones, ampicillin for Destination clones). The *E. coli* strain DB3.1 is

resistant to the effects of the *ccdB* gene and is used to propagate Gateway vectors in the presence of chloramphenicol.

Using the Gateway technique, libraries of Entry clones have been generated and derivatized to Destination clones for use in high-throughput studies. For example, the approximately 12,000 full-length ORFs available in the *Caenorhabditis elegans* ORFeome (Reboul et al. 2003) have been transferred to yeast two-hybrid vectors to determine a network of protein–protein interactions (Li et al. 2004). Similarly, many of the 6000 promoters in the *C. elegans* Promoterome were cloned upstream of GFP for gene expression analysis and for transcription regulatory network mapping by high-throughput yeast one-hybrid assays (Dupuy et al. 2004; Deplancke et al. 2006; Vermeirssen et al. 2007). Any reporter construct can be adapted to take advantage of Gateway Entry clone collections (see Box 1). Recent experimental techniques that were made Gateway-compatible include RNA interference (RNAi) (Rual et al. 2004) and protein-binding microarrays (Grove et al. 2009).

Disadvantages of Gateway Cloning and Alternative Cloning Systems

Gateway cloning works extremely efficiently and robustly, with one researcher able to generate hundreds of constructs in a matter of days. The major disadvantage of the system is that once the conversion is made to Gateway cloning, it is quite challenging to switch to another recombination system or to go back to traditional cloning methods (e.g., because start/stop codons have often been removed and Gateway vectors lack convenient restriction endonuclease sites). Another limitation is that Gateway vectors and recombination enzymes are quite expensive. However, in our experience, the cost–benefit ratio becomes favorable once the number of reporter constructs required reaches several dozen. The remaining issues are technical, will differ among desired constructs, and largely concern the introduction of the *att* sites (the *att* sites could, e.g., potentially change the functionality of the resulting protein fusion or add *cis*-regulatory sites to a promoter fragment). However, we have previously shown that the *att* sites are unlikely to interfere with yeast one- and two-hybrid assay readouts (Walhout et al. 2000; Deplancke et al. 2004). For each downstream assay, appropriate controls should be used to determine if the *att* sites affect the results. Finally, Gateway Destination vectors can be reconfigured if necessary, for example, to generate free protein end(s) by moving the fusion from the amino-terminal to the carboxy-terminal end or by introducing a new start/stop codon such that the *att* sites are not translated.

The Gateway cloning system is not the only recombination-based option for generating reporter constructs. The Univector (Liu et al. 1998) and Creator (Siegel et al. 2004) systems both use Cre recombinase from bacteriophage P1 (Abremski and Hoess 1984) that catalyzes recombination at *loxP* sites, whereas the In-Fusion (Berrow et al. 2007), MAGIC (Li and Elledge 2005), and SEFC (Zhu et al. 2009) systems use homologous recombination either in vitro or within *E. coli* strains. All of these alternative cloning systems have been used to generate large libraries of reporter constructs. Cre-based systems are superficially similar to Gateway, but the lack of variety of recombination sites and Destination vectors limits experimental options. The major advantages of homologous recombination are (i) that it does not require the purchase of recombinase enzymes and (ii) that no recombination sites are used that may interfere with the experiment. However, a significant disadvantage of this

approach is that transfer reactions are not always precise, and, thus, sequence confirmation of final constructs is required. Other advantages and disadvantages of each cloning system are listed in Table 1.

The website for Life Technologies (Invitrogen, <http://www.invitrogen.com>), the commercial source of all Gateway cloning reagents, features comprehensive protocols (and further background information) for the Gateway system. The following associated protocols are the most common protocols used in Gateway cloning. The first associated protocol describes how to propagate Gateway vectors that contain functional Gateway cassettes (see Protocol: **Propagating Gateway Vectors** [Reece-Hoyes and Walhout 2018a]); two additional protocols involve manipulating these vectors with Gateway recombination enzymes to generate Entry and Destination Clones. Of these, one protocol describes how to generate an ORF Entry clone using BP cloning, followed by LR transfer of the ORF into a Destination vector (see Protocol: **Generating an Open Reading Frame (ORF) Entry Clone and Destination Clone** [Reece-Hoyes and Walhout 2018b]). The final protocol describes a multisite Gateway reaction during which a promoter and ORF are transferred simultaneously from two different Entry clones into the same Destination vector using LR enzymes (see Protocol: **Using Multisite LR Cloning to Generate a Destination Clone** [Reece-Hoyes and Walhout 2018c]). All three of these protocols make use of specific vectors and transfer reactions; however, these steps can be applied to any Gateway vector and transfer reaction simply by substituting details specific to the required vector, such as *att* sites, selection antibiotic, and recombinase enzyme mix.

Acknowledgments

This work was funded by National Institutes of Health (NIH) grants DK068429 and GM082971 to A.J.M.W.

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Box 1**Generating Gateway-Compatible Vectors**

Making your desired reporter vector Gateway-compatible involves using conventional restriction endonucleases and ligation to clone a Gateway cassette flanked by att sites into the appropriate position within the vector backbone and then transforming and propagating the circular product in the DB3.1 E. coli strain. Because this new plasmid will function as a Destination vector that receives an insert from an Entry clone, it is important to use att sites that are compatible (e.g., if your vector needs to accept ORFs from pDONR 221 clones, then it must have attR1–attR2 sites). Depending on the vector, it may also be important to clone the att sites in a particular frame (i.e., so that every recombined ORF is expressed in-frame with an amino- or carboxy-terminal fusion). Blunt-ended DNA fragments containing the Gateway cassette flanked by attR1 and attR2 sites are commercially available in all three frames for ligation into your vector of interest. If different att sites are required, similar fragments can be amplified from existing vectors using PCR. If no vectors are available with the required att sites, existing att sites can be modified using PCR-mediated mutagenesis.

Figure 1.

Generating an Entry clone using a Gateway BP reaction. (A) A Gateway BP reaction uses BP clonase enzymes to recombine an *attP* site with an *attB* site to generate an *attL* site and an *attR* site, whereas LR clonase performs the reverse recombination. (B) *attP*, *attL*, and *attR* sites have recognition “arms” on either/both sides of the “recognition region” (box with arrowhead), whereas *attB* sites have no arms. These arms contain binding sites for the recombination enzymes and are named “left” and “right” with respect to the asymmetric overlap: *attL* sites have an arm 5′ (or left) of the overlap (white box), *attR* sites have an arm 3′ (or right, blue box), and *attP* sites have both arms. (C) *attB* sites are ~25 bp and feature a central 7-bp “asymmetric overlap” (boxed) that determines where DNA is cut and rejoined. *attB0* is the naturally occurring site in the *E. coli* genome that is used by bacteriophage λ (Hartley et al. 2000). Mutagenesis has been used to generate the other varieties of *attB* sites.

Figure 2.

Generating an Entry clone using a Gateway BP reaction. The creation of Entry clones, shown here, involves propagating a Donor vector in DB3.1 bacteria, amplifying a polymerase chain reaction (PCR) product with compatible *attB* sites, and then setting up a Gateway BP reaction. The BP clonase enzymes recombine the *attB* and *attP* sites, replacing the Gateway cassette with the amplified insert, which is now flanked by *attL* or *attR* sites depending on the configuration of the DNA fragments and vectors. The reaction shown on the *left* generates a promoter Entry clone, whereas that shown on the *right* generates an ORF Entry clone. Note that unidirectional cloning is ensured because *attB*1 sites only recombine with *attP*1 sites, *attB*2 sites with *attP*2 sites, and so on. After the BP reaction mixture is transformed into a standard bacterial cloning strain (such as DH5α), only the Entry clone will confer growth. Note that, typically, the asymmetric overlap is oriented 5′–3′ toward the insert, but *att* sites can also function in the reverse orientation and are then given the designation “R” (e.g., *attB*1R and *attP*1R). These reverse *att* sites are important for generating Entry clones that can be used in multisite LR reactions (Fig. 4).

Figure 3.

Generating a Destination clone using a Gateway LR reaction. In a Gateway LR reaction, an Entry clone is mixed with a Destination vector, and the LR clonase enzymes recombine the *attL* and *attR* sites of the matching subtype (i.e., *attR*4 with *attL*4, *attR*1 with *attL*1), swapping the Gateway cassette with the cloned insert. The reaction at *left* generates a reporter construct with a promoter cloned upstream of a reporter such as GFP or luciferase. The reaction at *right* generates a reporter construct with an ORF cloned in-frame with an amino-terminal reporter such as GST or the yeast two-hybrid moieties (AD or DB).

Figure 4.

Multisite Gateway LR reactions. In a multisite Gateway LR reaction, two Entry clones are mixed with a single Destination vector, and, just as in the LR reaction shown in Figure 3, the LR clonase enzymes recombine the matching subtypes of *attL* and *attR* sites. However, the result of this type of recombination is that the inserts from the two Entry clones (e.g., a promoter and ORF) are fused together when replacing the Gateway cassette.

Table 1
Advantages and disadvantages of different recombination-based cloning methods

Cloning system(s)	Method	Advantages	Disadvantages
Gateway	λ -based BP and LR recombinases that use <i>att</i> sites Completely in vitro	No gain/loss of nucleotides during transfer No digestion required Recombination-reversible Uses Entry clones Dozens of Destination vectors available Multisite transfers possible Wide variety of recombination sites	Need to purchase enzymes Recombination sites may interfere with experiment Need to make desired vector compatible
Univector Creator	P1-based Cre recombinase that uses <i>loxP</i> sites Completely in vitro	No gain/loss of nucleotides during transfer No digestion required Uses Entry clones	Need to purchase enzymes Recombination sites may interfere with experiment Recombination irreversible Few Destination vectors available Multisite transfers not possible Limited variety of recombination sites Need to make desired vector compatible
In-Fusion, MAGIC, SEFC	Homologous recombination ^a	No recombination sites No enzyme purchase necessary ^a Multisite transfers possible Any vector can be used without conversion	Occasional gain/loss of nucleotides during transfer Need to generate insert by either PCR or digest before recombination Does not use Entry clones ^b

^aIn-fusion is an in vitro system that requires purchase of enzymes, whereas for MAGIC and SEFC recombination occurs within *E. coli* strains.

^bIt is possible to have an Entry clone-type setup in the MAGIC system.