Chapter 3

Gateway Cloning for Protein Expression

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Summary

The rate-limiting step in protein production is usually the generation of an expression clone that is capable of producing the protein of interest in soluble form at high levels. Although cloning of genes for protein expression has been possible for some time, efficient generation of functional expression clones, particularly for human proteins, remains a serious bottleneck. Often, such proteins are hard to produce in heterologous systems because they fail to express, are expressed as insoluble aggregates, or cannot be purified by standard methods. In many cases, researchers are forced to return to the cloning stages to make a new construct with a different purification tag, or perhaps to express the protein in a different host altogether. This usually requires identifying new cloning schemes to move a gene from one vector to another, and frequently requires multistep, inefficient cloning processes, as well as lengthy verification and sequence analysis. Thus, most researchers view this as a linear pathway – make an expression clone, try it out, and if it fails, go back to the beginning and start over. Because of this, protein expression pipelines can be extremely expensive and time consuming.

The advent of recombinational cloning has dramatically changed the way protein expression can be handled. Rapid production of parallel expression clones is now possible at relatively low cost, opening up many possibilities for both low- and high-throughput protein expression, and increasing the flexibility of expression systems that researchers have available to them. While many different recombinational cloning systems are available, the one with the highest level of flexibility remains the Gateway system. Gateway cloning is rapid, robust, and highly amenable to high-throughput parallel generation of expression clones for protein production.

Key words: Recombinational cloning; Gateway; Cloning; Protein expression; HTP cloning; Site-specific recombination; *att* sites; Fusion proteins; Solubility tags

3.1. Introduction

The Gateway recombinational cloning system is a robust method for generating a wide variety of protein expression constructs for use in multiple host systems *(1)*. It also has the advantage of

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being readily scalable from a single construct cloning strategy to a high-throughput system for generating large numbers of parallel clones. In both cases, the more thorough the development of the initial cloning strategy is, the better the chances are for success downstream. We find that in many applications, increasing the work slightly at the initial cloning stage produces exponential increases in the downstream value of the clones generated. The choices which investigators need to make upfront are the focus of the discussion in the sections that follow. We have chosen to examine two possible scenarios that should benefit the largest number of researchers. First, there is the "low-throughput" scenario in which a single protein of interest is to be examined in either multiple host systems or with multiple fusion tags. Second, there is the "high-throughput" scenario, in which a large number of proteins (96 is used as a convenient example here) are expressed in the same host and under the same conditions. These two scenarios provide a range of methods that cover most possibilities for the use of Gateway cloning for protein production, and the similarities and differences in the methods for both will be highlighted later.

Gateway cloning is a multistep recombinational cloning method which eliminates the need for classical restriction enzyme and ligase (REaL) cloning for the transfer of genes between vectors. The parallel power of Gateway cloning involves the transfer of a gene of interest into many different expression vectors through simple recombination reactions which maintain protein reading frame (*see* **Fig. 3.1** for an overview). Two types of clones are generated using Gateway: Entry clones, which are transcriptionally silent "master" clones that are sequence verified, and Expression clones, which are the final protein production clones generated by recombination of the Entry clones. Because the Gateway site-specific recombination reaction does not involve PCR amplification, Expression clones do not need to be resequenced as long as their parent Entry clones have been sequence verified. For this reason, a large number of Expression clones can be generated easily from a single Entry clone without the need for additional amplification and sequencing. Gateway reactions are driven by recombination between sites called attachment sites (*att* sites), which come in four varieties, *attP*, *attB*, *attL*, and *attR*. All reactions are conservative, directional, and lead to the interconversion of these sites, the types of which can be used to differentiate the vectors. Entry clones contain *attL* sites, while Expression clones contain *attB* sites. The core recombination site in all these *att* sites is the same 21-bp DNA sequence which determines the directionality and specificity of the reaction. The protein-coding sequences of Gateway clones are always flanked by two slightly different *att* sites, identified with numbers as in *attB*1 and *attB*2. The *att*1 and *att*2 sites are unable to recombine with each other, and thus produce the unique order of the

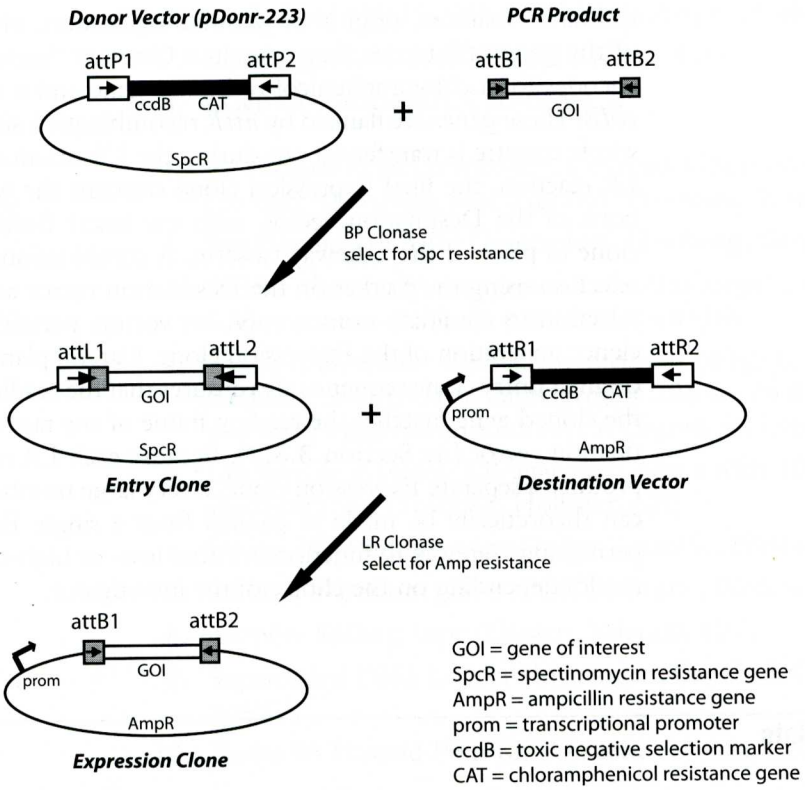

Fig. 3.1. General Gateway cloning scheme. This diagram presents a schematic representation of the standard Gateway reactions, identifying the different plasmid types found in the BP and LR reactions along with the relevant selection markers.

recombination reactions that maintains the reading frame and eliminates the need for directional screening.

Genes of interest can be inserted into Entry clones in several different ways. In order to provide most flexibility in the cloning strategy, we generally use a Gateway recombination reaction (called the BP reaction, *see* **Section 3.3.4**) to transfer genes into Entry clones. Additional entry routes are available, including traditional REaL cloning into commercially available Entry vectors, Topo cloning, or purchase of prepared Gateway ORFeome clones from suppliers such as Open Biosystems. In some cases, these methods are acceptable ways of obtaining Entry clones; however, the flexibility of adding epitope or purification tags is often lost in this process, making the recombination route much more useful. Once an Entry clone is constructed and sequence-verified, a second recombination reaction (called an LR reaction, *see* **Section 3.3.5**) is used to transfer the gene of interest from the Entry clone to the final Expression clone. The vectors used in the transfer are called Destination vectors (DVs). The DVs contain all the signals needed to make protein in the host of interest, including promoters,

resistance markers, origins of plasmid replication, etc. In place of the gene of interest, they contain a Gateway "cassette" which encodes for a chloramphenicol resistance gene and a toxin called *ccdB*. These genes are flanked by *attR* recombination sites, and this whole cassette is transferred out during the LR reaction. After the LR reaction, the final Expression clone contains the whole backbone of the Destination vector, with the insert from the Entry clone in place of the Gateway cassette. A combination of positive selection using the marker on the Destination vector and negative selection to eliminate nonrecombinant vectors permits high efficiency generation of the Expression clone. Careful planning of the original Entry clone sequence also ensures that the reading frame of the cloned gene matches the reading frame of any tags in the Destination vector (*see* **Section 3.3.2**). Because each LR reaction can produce a separate Expression clone, a very large number of clones can theoretically be made in parallel from a single Entry clone, permitting Gateway to function in either low- or high-throughput modes depending on the choice of the investigator.

3.2. Materials

3.2.1. Determining Protein Context

1. TEV protease can be produced by published methods *(2)* using the pRK793 expression vector available from Addgene (http://www.addgene.org)
2. Gateway Destination Vector Conversion Kit (Invitrogen, Carlsbad, CA)

3.2.2. Oligonucleotide Design

1. Oligonucleotides can be ordered from numerous suppliers, such as Operon (Germantown, MD). We have found that they generally do not require HPLC 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.
2. Oligonucleotides for PCR amplification should be resuspended to a concentration of 10 µM in TE (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA).

3.2.3. PCR Amplification

1. 2× Phusion Master Mix HF (New England Biolabs, Beverley, MA)
2. DMSO: dimethyl sulfoxide (provided with Phusion Kit or available from Sigma, St. Louis, MO)
3. 96-Well PCR Plates (PGC Scientific, Frederick, MD)
4. QiaQuick PCR Purification Kit (Qiagen,Valencia, CA)
5. QiaQuick 96 PCR Purification Kit (Qiagen, Valencia, CA)

6. Ready-Load 1 kB Plus DNA Ladder (Invitrogen, Carlsbad, CA)
7. ReadyAgarose Gels (BioRad, Hercules, CA)
8. EGel-96 (Invitrogen, Carlsbad, CA)

3.2.4. BP Recombination

1. BP Clonase II kit (Invitrogen, Carlsbad, CA; comes with BP Clonase® II enzyme mix, 2 µg/mL Proteinase K solution).
2. FastPlasmid DNA Kit (Eppendorf, Hamburg, Germany).
3. DH5α chemically competent cells (Invitrogen, Carlsbad, CA). Store at –80°C, do not reuse open vials.
4. CG-Spec medium: Circlegrow medium (40 g/L, MP Biomedicals, Solon, OH), autoclave for 20 min, cool to 55°C, and add 50 µg/mL spectinomycin (Sigma, St. Louis, MO).
5. LB-Spec agar plates: LB-agar petri plates with 100 µg/mL spectinomycin (Teknova, Hollister, CA).
6. Falcon 2059 culture tubes (Fisher Scientific, Pittsburgh, PA).
7. 96-Well V-bottom plates (PGC Scientific, Frederick, MD).
8. Air-pore Sealing Tape (Qiagen, Valencia, CA).
9. Supercoiled DNA Ladder (Invitrogen, Carlsbad, CA). Store at 4°C.
10. Turbo 96 Plasmid Prep Kit (Qiagen, Valencia, CA).

3.2.5. LR Recombination

1. LR Clonase II enzyme mix (Invitrogen, Carlsbad, CA; comes with LR Clonase® II enzyme mix, 2 µg/mL Proteinase K solution.)
2. *E. coli ccdB* Survival competent cells (Invitrogen, Carlsbad, CA). Store at –80°C, do not reuse open vials.
3. *BsrG*I restriction enzyme (New England Biolabs, Beverley, MA).

3.2.6. Downstream Applications

1. GenElute Midiprep DNA Kit (Sigma, St. Louis, MO).
2. *E. coli* DH10Bac competent cells (Invitrogen, Carlsbad, CA). Store at –80°C, do not reuse open vials.
3. LB-KGTXI agar plates: LB-agar petri plates with 7 µg/mL gentamycin, 50 µg/mL kanamycin, 10 µg/mL tetracycline, 40 µg/mL IPTG, and 100 µg/mL Bluo-gal (Teknova, Hollister, CA).
4. CG-KG medium: Circlegrow medium (40 g/L, MP Biomedicals, Solon, OH), autoclave for 20 min, then add 50 µg/mL kanamycin and 7 µg/mL gentamycin (Sigma, St. Louis, MO).
5. LB-agar petri plates (Teknova, Hollister, CA).
6. Benzonase Cracking Buffer (BCB): 25 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$. Store at room temperature.
7. Benzonase (EMD Biosciences, Darmstadt, Germany).

8. NuPAGE 4× LDS Sample Buffer (Invitrogen, Carlsbad, CA). Store at room temperature.
9. TCEP: Tris(2-carboxyethyl)phosphine hydrochloride (Pierce, Rockford, IL). Store at room temperature. Do not use for more than 3 months after opening.
10. MDG medium: a defined medium for growth of *E. coli* under conditions which will not induce T7 polymerase production. For detailed recipes, *see (3)*.
11. Plasmid Prep Buffer A: 25 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.9% D-glucose. Store at room temperature.
12. Plasmid Prep Buffer B: 0.20 M NaOH, 1.33% sodium dodecyl sulfate (SDS). Store at room temperature. SDS may precipitate at low temperatures – redissolve before use by heating at 37°C. Make fresh after 6 months, and be sure to cap immediately after use.
13. Plasmid Prep Buffer C: 7.5 M ammonium acetate (heat to 50°C to dissolve). Store at room temperature.
14. Plasmid Prep Resuspension Buffer: 10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 0.1 µg/mL ribonuclease A (Sigma, St. Louis, MO). Store at room temperature for up to 6 months.
15. Benchmark Protein Ladder (Invitrogen, Carlsbad, CA).
16. Rosetta(DE3) competent cells (Novagen, La Jolla, CA). Other BL21(DE3) varieties can also be purchased as competent cells from Novagen.
17. Bac-to-Bac Baculovirus Expression Kit (Invitrogen, Carlsbad, CA).
18. Criterion SDS–PAGE gels (BioRad, Hercules, CA).
19. 10× TGS Buffer: 250 mM Tris base, 1.92 M glycine, 1% SDS (USB, Cleveland, OH).
20. GelStain: mix 175 mg Brilliant Blue G (Sigma, St. Louis, MO) into 10.5 mL acetic acid and add water to a final volume of 3.5 L. Stir for 15 min on a magnetic stirplate, and filter the solution over Whatman #1 filter paper. Some precipitation may be observed over time, and stain should not be stored for more than 3 months without refiltering.

3.3. Methods

3.3.1. Determining Protein Context

1. Prior to the start of any cloning project, a determination of the desired protein context must be made in order to maximize the downstream flexibility of the final expression clones. Proper choices at this stage can save time and money

later when expression may fail or be unacceptably low under certain conditions.

2. Gateway recombination replaces sequences between the *att* sites on a given plasmid with the sequences between the *att* sites on the other plasmid. For this reason, sequences such as fusion tags, translational start sites, etc. can be placed either in the Entry clone sequence between the *att* sites, or on the Destination Vector outside the *att* sites. Both will produce final Expression clones that make the protein of interest, but the difference in protein sequence may have experimental relevance.
3. For projects requiring the most flexibility, we suggest minimizing the amount of extra sequence present in the Entry clone. The simplest such construct would be an Entry clone containing only the gene of interest with a stop codon. Such a clone can be used to make aminoterminal fusions by subcloning to a Destination vector with an aminoterminal tag. Readthrough of the *attB*1 site would provide an additional 7–8 amino acids between the tag and the protein, but often this is not of major consequence for fusion proteins.
4. If near-native sequence is required, the simplest alteration to this scheme is to include a protease cleavage site in the Entry clone prior to the start of the gene of interest. Our preferred protease of choice is the Tobacco Etch Virus (TEV) protease, which very specifically cleaves a sequence ENLYFQ/X, where X is any amino acid except for proline *(2)*. The addition of this sequence to the Entry clone will permit native protein to be cleaved from any aminoterminal fusion generated by LR recombination (*see* **Note 1**).
5. In many cases, epitope or purification tags are necessary for steps in protein production. Often, multiple tags are necessary to permit multiple rounds of purification or detection. A common technique is to include a protease cleavage site at the start of the gene, and to add an epitope tag (FLAG, HA, His6) to the end of the gene prior to the stop codon. To avoid adding the 7–8 amino acids of the *attB*2 site to such a construct, it is best to add this tag inside the Entry clone sequence. Such small epitope tags are readily added in the PCR amplification step (*see* **Sections 3.3.2** and **3.3.3**).
6. Additional sequences may be required depending on the specific case. A common motif for prokaryotic expression is the addition of a Shine-Dalgarno translation initiation site *(4)*, which should be placed inside the Entry clone for maximal utility. Note, however, that such a sequence will eliminate the possibility of making aminoterminal fusions in bacteria, as all aminoterminal fusion Destination vectors carry their own Shine-Dalgarno sequence, which would lead to translation initiation at two sequences and a mixture of proteins.

Table 3.1
Common Entry clone sequence contexts

GOI-stop	Aminoterminal fusions
GOI-nostop	Aminoterminal and/or carboxyterminal fusions
Kozak-GOI-stop	Aminoterminal fusions or native eukaryotic expression
TEV-GOI-stop	Cleavable aminoterminal fusions
TEV-GOI-Tag	Cleavable am inoterminal fusions with carboxyterminal epitope/purification tag
SD-GOI-stop	Native expression in *E. coli*
Tag-GOI-stop	Aminoterminal tag inside the Entry clone

GOI gene of interest; Kozak, eukaryotic translation initiation sequence; TEV, TEV protease cleavage site; SD, bacterial translation initiation site; Tag, any small epitope or purification tag, such as His6 or FLAG

7. **Table 3.1** provides a list of common motifs for Entry clones which we and others have used. In most cases, these will suffice for wide flexibility. In some cases, additional sequences will be required that are too large for facile introduction into Entry clones. Such sequences (e.g., large fusion tags) can then be placed inside the Destination vectors instead (*see* **Note 2**).

3.3.2. 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 on to the gene of interest. Proper design of the oligonucleotides for this step ensures that the proper reading frame is generated in the final expression clone.
2. To clone most genes, 18–21 bp of gene-specific 5′ and 3′ sequences are used for primer annealing. For simple Entry clones which do not contain large amounts of additional features, the recombination signal sequences given in **Table 3.2** (*attB*1 at the 5′ end of the gene, and *attB*2 at the 3′ end) can be added directly to the gene-specific primers.
3. Longer 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 bp. In our experience, such long oligonucleotides often are of reduced quality containing higher numbers of mutations or deletions. This requires more clones to be sequenced in order to avoid frameshift errors. To avoid this problem, a technique known as adapter PCR can be utilized.

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 which contains the gene-specific portion and part or all of a tag sequence (such as a TEV protease site) is added to the PCR. After a few rounds of amplification, a second primer is added which contains the *attB* recombination signal and 12–18 bp of overlapping sequence with the first primer. 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. **Table 3.2** shows some common adapter primer sequences and their corresponding gene-specific primers.
5. Adapter PCR can be used on both ends simultaneously by adding two different adapter primers. One can also nest multiple levels of adapter PCR to insert long 5′ or 3′ sequences if necessary

Table 3.2
Oligonucleotide sequences for Gateway sites and some common adapters

Standard Gateway primers	
*attB*1 (no Kozak)	5′-GGGGACA ACT TTG TAC AAA AAA GTT GGC – gsp
*attB*1 (Kozak-ATG)	5′-GGGGACA ACT TTG TAC AAA AAA GTT GGC ACC ATG – gsp
*attB*2 (stop)	5′-GGGGAC AAC TTT GTA CAA GAA AGT TGG *CTA* – gsp (reverse comp)
*attB*2 (stop)	5′-GGGGAC AAC TTT GTA CAA GAA AGT TGG – gsp (reverse comp)
Sequences for initial PCR of genes with additional sequences	
Amino-TEV	5′-GGC GAA AAC CTG TAC TTC CAA GGC – gsp
Amino-SD	5′-TTT AAC TTT AAG AAG GAG ATA TAT ACC ATG – gsp
Amino-His6	5′-ACC ATG TCA CAC CAT CAC CAT CAC CAT – gsp
Carboxy-His6	5′-GT TGG *CTA* ATG GTG ATG GTG ATG GTG ACC – gsp
Gateway adapter primers	
*attB*1-Tev adapter	5′-GGGGACAACTTTGTACAAAAAAGTTGGCGAAAAC CTGTACTTCCAAGGC
*attB*1-SD adapter	5′-GGGGACAACTTTGTACAAAAAAGTTGGCTCATT-TAACTTTAAGAAGG
*attB*1-aminoHis6 adapter	5′-GGGGACAACTTTGTACAAAAAAGTTGGCACCAT GTCACACCATCACCATCACCAT
*attB*2-carboxyHis6 adapter	5′-GGGGACAACTTTGTACAAGAAAGTT G*GCTA*ATGGTGATGGTGATGGTGACC

gsp gene-specific primer (should contain 18–21 bp of 5′ or 3′ of gene, in *attB*2 primers this sequence must be the reverse complement of the sense strand of the gene). *Underlined* sequences in the *att* sites identify the proper reading frame (*see* **step 7 – Section 3.3.2**). Stop codons in *attB*2 sequences are *italicized*

(*see* **Note 3**). Often, the same adapter primers can be used for any gene which has a particular 5′ sequence (i.e., the TEV adapter listed in **Table 3.2**), thus minimizing the cost and number of oligos which need to be generated for a project.

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 *(5)*. 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 two 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 (*see* **Note 4**).
7. In order to maintain the proper reading frame for Gateway fusions (both amino and carboxy ends), care must be taken in the design of oligonucleotides. Key DNA sequences are present in both the *attB*1 and the *attB*2 sequences to guide reading frame alignment. As shown in **Table 3.2**, the *attB*1 reading frame is centered on the 2 lysine codons encoded by the 5′-AAA AAA sequence. For a gene to be in frame, it must be aligned properly with this sequence. The *attB*1 oligonucleotide sequence given in **Table 3.2** has an additional 3′ nucleotide not actually required for recombination in order to ensure the reading frame is intact. Likewise, the *attB*2 reading frame is centered on the 5′-TTT GTA sequence, and those codons must be in the same frame as the gene of interest (*see* **Note 5**).
8. In addition to oligonucleotides for amplification, oligonucleotides for sequence verification of Entry clones are also required. We generally order 1 primer for every 600 bp of sequence, and an additional primer on the reverse strand that is able to sequence back through the start of the gene. Typical primer lengths are 22–24 bp, 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 (*see* **Note 6**).

3.3.3. PCR Amplification

3.3.3.1. Single Protein PCR Amplification

1. To a 200-µL thin-walled PCR tube, add 1 µL of each 10 µM oligonucleotide primer, 1.5 µL DMSO (*see* **Note 7**), 100–200 ng template DNA (*see* **Note 8**), and water to 25 µL final volume.
2. Add 25 µl 2× Phusion Master Mix HF, mix well, and carry out the PCR amplification using the following parameters: initial denaturation at 98°C for 30 s, 20 cycles of (98°C for 10 s, 55°C for 30 s, and 72°C for 30 s per kB of the expected

product), followed by a 10-min final elongation at 72°C, and cooling to 4°C (*see* **Note 9**).

3. For adapter PCR (*see* **step 5 – Section 3.3.2**), after 5 cycles of amplification, pause the thermal cycler, and add 1 µL of 10 µM adapter primer(s) to the tubes. Continue cycling for an additional 15 cycles.
4. If multiple nested adapter primers are used, we suggest increasing the overall cycle time so that there are 5 cycles between adapter additions, and at least 15 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 1% agarose gel to verify size by comparison to a linear DNA standard such as the ReadyLoad 1 kB DNA ladder (*see* **Note 10**).
7. Purify the PCR product using the QiaQuick PCR Purification Kit following the manufacturer's protocol and elute the DNA in 50 µL (*see* **Note 11**).

3.3.3.2. High-Throughput PCR Amplification

1. To each well of a 96-well PCR plate, add 0.5 µL of each 10 µM oligonucleotide primer, 1 µL DMSO (*see* **Note 7**), 50–100 ng template DNA (*see* **Note 8**), and water to 12.5 µL final volume. Addition of primers will be simplified if the oligonucleotides are also in a 96-well format, permitting addition by multichannel pipetting. A master mix of DMSO and water, as well as primers or template, can also be employed where applicable.
2. Add 12.5 µl 2× Phusion Master Mix HF per well, mix well, and carry out the PCR amplification using the following parameters: initial denaturation at 98°C for 30 s, 20 cycles of (98°C for 10 s, 55°C for 30 s, and 72°C for 30 s per kB of the expected product), followed by a 10-min final elongation at 72°C, and cooling to 4°C (*see* **Note 9**).
3. For adapter PCR (*see* **step 5 – Section 3.3.2**), after 5 cycles of amplification, pause the thermal cycler, and add 0.5 µL of 10 µM adapter primer(s) to the tubes. Continue cycling for an additional 15 cycles.
4. If multiple nested adapter primers are used, we suggest increasing the overall cycle time so that there are 5 cycles between adapter additions, and at least 15 additional cycles after the final adapter is added.
5. After cycling, load 5 µL of the PCR product on a 1% agarose gel to verify size. It is recommended that a 96-well gel format be utilized at this step to reduce effort – options include the e-Gel 96 or various 96-well horizontal agarose gel platforms.

Verify size by comparison to a linear DNA standard such as the ReadyLoad 1 kB DNA ladder.

6. Purify the PCR products using the QiaQuick 96 PCR Purification Kit following the manufacturer's protocol and elute the DNA in 50 µL.

3.3.4. BP Recombination

3.3.4.1. Single Protein BP Recombination

1. Add the following reagents to a microcentrifuge tube in the order given (the total reaction volume should be 10 µL): 1–6 µL H_2O, *attB* flanked PCR fragment (15–150 ng, *see* **Note 12**), 150 ng pDonr-223 vector (*see* **Note 13**), 2 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 briefly by gentle vortexing.
2. Incubate the reaction mixture for at least 1 h at 30°C (*see* **Note 14**).
3. Add 1 µL of 2 mg/mL Proteinase K to inactivate the BP Clonase and incubate for 15 min at 37°C (*see* **Note 15**).
4. Add 1 µL of the BP reaction to a microcentrifuge tube containing 20 µL of chemically competent *E. coli* DH5 and incubate on ice for 5–10 min (*see* **Note 16**).
5. Heat shock the cells in 42°C water bath for 45 s and immediately add 80 µL of SOC medium. Shake the reaction for 1 h at 37°C.
6. Spread 50 µL of the transformation mix on LB-Spec agar plates and incubate overnight at 37°C. A good BP cloning result with a standard length (1 kB) gene should yield greater than 200 colonies per transformation (*see* **Note 17**).
7. Pick 2–4 separate Entry clone colonies into Falcon 2059 culture tubes containing 2 mL of CG-Spec media and grow overnight at 37°C with 200 rpm shaking.
8. Spin 1 mL of the culture in a microcentrifuge to pellet the cells, and isolate plasmid using the FastPlasmid kit, eluting the DNA in 75 µL of elution buffer (*see* **Note 18**).
9. Verify the size of the plasmid using agarose gel electrophoresis. Load 1 µL of the purified Entry clone DNA on a 1% agarose gel, and compare sizes to the Supercoiled DNA ladder.
10. Properly sized Entry clones should be sequence verified to ensure that no oligonucleotide or PCR-generated errors have been introduced.
11. Glycerol stocks of the *E. coli* strains containing Entry clones should be made by adding 250 µL sterile filtered 60% glycerol to 750 µL of culture. After mixing and incubation at room temperature for 5 min, these stocks can be frozen at −80°C and used to start new cultures if more Entry clone DNA is required in the future.

3.3.4.2. High-Throughput BP Recombination

1. Add the following reagents to a 96-well v-bottom plate in the order given (the total reaction volume should be 10 µL): 1–6 µL of H_2O, 50–100 ng *attB* flanked PCR fragment, 150 ng pDonr-223 vector, 2 µL of BP Clonase II. A master mix can be used for all reagents except for BP Clonase II, which must be added last.
2. Centrifuge the plate to mix the reaction thoroughly and incubate the reaction for at least 2 h at 30°C (*see* **Note 14**).
3. Add 1 µL of 2 mg/mL Proteinase K to inactivate the BP Clonase and incubate for 15 min at 37°C (*see* **Note 15**).
4. Add 1 µL of the BP reaction to a 96-well v-bottom plate containing 10 µL of chemically competent *E. coli* DH5α and incubate on ice for 5–10 min (*see* **Note 16**).
5. Heat shock the cells in a 42°C water bath for 45.0 s and immediately add 40 µL of SOC medium. Shake the reaction for 1 h at 37°C.
6. Spread the whole 50 µL of the transformation mix on LB-Spec agar plates and incubate overnight at 37°C.
7. Pick 1–2 separate Entry clone colonies into a 96-well deep well plate containing 1.5 mL of CG-Spec media per well, cover with Air-Pore sealing tape, and grow overnight at 37°C with 250 rpm shaking.
8. Isolate plasmid DNA using a commercially available high-throughput plasmid prep kit, such as the Qiagen Turbo 96 Kit (*see* **Note 19**).
9. Verify the size of the plasmids using agarose gel electrophoresis. Load 1 µL of the purified Entry clone DNA on a 1% agarose gel, and compare sizes to a supercoiled DNA ladder (Invitrogen, Carlsbad, CA).
10. Properly sized Entry clones should be sequence verified to ensure that no oligonucleotide or PCR-generated errors have been introduced.
11. Glycerol stocks of the *E. coli* strains containing Entry clones can be made by adding 50 µL sterile filtered 60% glycerol to the wells of a microtiter plate. Add 150 µL of Entry clone culture to each well, cover with aluminum sealing tape, and freeze at −80°C for long-term storage.

3.3.5. LR Recombination

3.3.5.1. Single Protein LR Recombination

1. Add the following reagents to a microcentrifuge tube in the order given (the total reaction volume should be 10 µL): 1–6 µL H_2O, Entry clone DNA (50–100 ng, *see* **Note 20**), Destination Vector DNA (150 ng, *see* **Note 21**), 2 µL of LR Clonase II.
2. Incubate the reaction mixture for at least 1 h at 30°C (*see* **Note 22**).

3. Add 1 µL of 2 mg/mL Proteinase K to inactivate the LR Clonase and incubate for 15 min at 37°C (*see* **Note 15**).
4. Add 1 µL of the LR reaction to a microcentrifuge tube containing 20 µL of chemically competent *E. coli* DH5α and incubate on ice for 5–10 min (*see* **Note 16**).
5. Heat shock the cells in 42°C water bath for 45 s and immediately add 80 µL of SOC medium. Shake the reaction for 1 h at 37°C.
6. Spread 50 µL of the transformation mix on LB agar plates containing the correct antibiotic (often ampicillin, but check the Destination vector literature) and incubate overnight at 37°C. A good LR cloning result should yield greater than 500 colonies per transformation.
7. Pick two separate Expression clone colonies into Falcon 2059 culture tubes containing 2 mL of CG media containing the necessary antibiotics and grow overnight at 37°C with 200 rpm shaking.
8. Spin 1 mL of the culture in a microcentrifuge to pellet the cells, and isolate plasmid using the FastPlasmid kit, eluting the DNA in 75 µL of elution buffer (*see* **Note 18**).
9. Verify the size of the plasmid using agarose gel electrophoresis. Load 1 µL of the purified Expression clone DNA on a 1% agarose gel, and compare sizes to the Supercoiled DNA ladder.
10. If additional confirmation of the Expression clone is required, restriction enzyme analysis can be performed (*see* **Note 23**).
11. Glycerol stocks of the *E. coli* strains containing Expression clones should be made by adding 250 µL sterile filtered 60% glycerol to 750 µL of culture. After mixing and incubation at room temperature for 5 min, these stocks can be frozen at −80°C and used to start new cultures if more Expression clone DNA is required in the future.

3.3.5.2. High-Throughput LR Cloning

1. Add the following reagents to a 96-well v-bottom plate in the order given (the total reaction volume should be 10 µL): 1–6 µL of H_2O, 50 ng Entry clone DNA, 150 ng Destination vector, 1 µL of LR Clonase II. A master mix can be used for all reagents except LR Clonase II, which must be added last.
2. Centrifuge the plate to mix the reaction thoroughly and incubate the reaction for at least 2 h at 30°C (*see* **Note 22**).
3. Add 1 µL of 2 mg/mL Proteinase K to inactivate the BP Clonase and incubate for 15 min at 37°C (*see* **Note 15**).
4. Add 1 µL of the LR reaction to a 96-well v-bottom plate containing 10 µL of chemically competent *E. coli* DH5α and incubate on ice for 5–10 min (*see* **Note 16**).

5. Heat shock the cells in a 42°C water bath for 45 s and immediately add 40 µL of SOC medium. Shake the plate for 1 h at 37°C.
6. Spread the whole 50 µL of each transformation mix on LB agar plates containing the correct antibiotic (often ampicillin, but check the Destination Vector literature) and incubate overnight at 37°C.
7. Pick one Expression clone colony from each plate into a 96-well deep well plate containing 1.5 mL of CG media plus antibiotics per well, cover with Air-Pore sealing tape, and grow overnight at 37°C with 250 rpm shaking.
8. Isolate plasmid DNA using a commercially available high-throughput plasmid prep kit, such as the Qiagen Turbo 96 Kit (*see* **Note 19**).
9. Verify the size of the plasmids using agarose gel electrophoresis. Load 1 µL of the purified Entry clone DNAs on a 1% agarose gel, and compare sizes to a supercoiled DNA ladder.
10. If additional confirmation of the Expression clone is required, restriction enzyme analysis can be performed (*see* **Note 23**).
11. Glycerol stocks of the *E. coli* strains containing Expression clones can be made by adding 50 µL sterile filtered 60% glycerol to the wells of a microtiter plate. Add 150 µL of Expression clone culture to each well, cover with aluminum sealing tape, and freeze at −80°C for long-term storage.

3.3.6. Downstream Applications

3.3.6.1. Preparation of Mammalian/Yeast Expression Clones

1. Generally for mammalian transfection or yeast transformation, large quantities of expression clone DNA are required. In these cases, a 50-mL culture of the expression clone should be grown using CG media with the proper selective antibiotics. Plasmid preparation from this culture can be accomplished using commercially available Midiprep DNA kits. We commonly use GenElute kits, which are rapid, inexpensive, and produce high-quality DNA (*see* **Note 24**).
2. For yeast transformation, expression clone DNA will sometimes require linearization with a restriction enzyme prior to use. This depends on the expression vector being used, and this information can usually be found in the literature that comes with the chosen vector.

3.3.6.2. Preparation of Baculovirus Expression Clones

1. Baculoviruses are used to express proteins in insect cells, which are often a more reliable host for the production of eukaryotic proteins. Numerous Gateway baculovirus vectors are available, and most use the Bac-to-Bac system for generation of recombinant baculoviruses *(6)*.
2. Transform Baculovirus Expression clones into *E. coli* DH10Bac cells using the following protocol: mix 1 µL of Expression

clone DNA and 50 µL of competent cells in a Falcon 2059 tube, and incubate on ice for 5 min. Heat shock samples at 42°C for 45 s, and add 450 µL SOC medium to the tube. Incubate tubes at 37°C for 4 h with 200-rpm shaking.

3. After growth, plate 5 and 25 µL of the culture on LB-KGXTI agar plates and incubate the plates overnight at 37°C. Restreaking of positive clones may be required on the following day (*see* **Note 25**).
4. Pick positive white colonies into 2 mL LB-KG medium and grow overnight at 37°C with 200 rpm shaking. One milliliter of this culture can be used to prepare bacmid DNA using a standard alkaline lysis plasmid preparation procedure as follows (*see* **Note 26**).
5. Centrifuge 1 mL of culture in a microcentrifuge tube at maximum speed for 1 min, and aspirate the supernatant.
6. Add 150 µL Plasmid Prep buffer A and mix by pipetting.
7. Add 150 µL Plasmid Prep buffer B and mix by gentle inversion.
8. Add 150 µL Plasmid Prep buffer C and mix by gentle inversion. A white flocculent precipitate will form.
9. Centrifuge for 10 min at maximum speed, and transfer 400 µL sample to a new microcentrifuge tube. Centrifuge for an additional 5 min at maximum speed and transfer 350 µL to a new microcentrifuge tube.
10. Add 800 µL of 100% ethanol, mix by vortexing.
11. Centrifuge for 20 min at maximum speed, decant supernatant, recentrifuge briefly, and pipet out the remaining liquid.
12. Dry the tubes at 37°C for 5 min with the caps open to evaporate any remaining ethanol.
13. Resuspend in 50 µL Plasmid Resuspension buffer, incubate for 5 min at 37°C.
14. Centrifuge at maximum speed for 2 min and transfer the supernatant to a new tube, avoiding transfer of any solid pellet or particulates.
15. Generally, 5–10 µL of this material can be used for insect cell transfections. PCR or gel analysis can be performed to verify the bacmid DNA if desired (*see* **Note 27**).

3.3.6.3. Expression in Escherichia Coli

1. Expression clones should be transformed into an appropriate *E. coli* strain using chemical transformation (*see* **Section 3.3.4.1, steps 4–6**), and plated on LB-agar plates containing the proper selective antibiotics (*see* **Note 28**).
2. After overnight incubation at 37°C, pick a single colony using a sterile toothpick or pipet tip, and inoculate it into 2-mL liquid growth media containing the proper antibiotics

for selection. For clones with a T7 promoter, we suggest MDG media to avoid induction in the initial growth phase. For other promoters, standard LB or CG media can be substituted.

3. Incubate starter cultures for 12–16 h at 37°C with 200-rpm shaking.
4. Dilute starter cultures 1:100 into fresh growth media (CG or equivalent rich medium is preferred) containing the proper antibiotics for selection, and grow at 37°C with 200 rpm shaking until an OD_{600} of 0.4–0.6 is reached. For most clones, this will take between 2 and 3 h.
5. Induce cells for protein expression with the appropriate inducer for the particular vector – in most cases this will be IPTG at a final concentration of 0.5–1.0 mM. After induction, continue to grow the cells for either 3–4 h at 37°C, or alternately for 16–20 h at 18°C (*see* **Note 29**).
6. After induction, remove a sample of the culture for SDS–PAGE analysis of induced proteins. Generally, it is a good idea to remove a sample prior to induction as well in order to compare the uninduced and induced cells. 0.1 OD units (100 µL of a culture with an OD_{600} of 1.0) is usually appropriate for gel analysis.
7. Centrifuge the cells in a microcentrifuge at maximum speed, and aspirate the supernatant. Resuspend cells in 20 µL Benzonase cracking buffer (BCB), mix, and freeze on dry ice for at least 5 min.
8. Thaw the frozen cell pellet and add 1 unit of Benzonase, and incubate at 37°C for 10 min. After incubation, add 7.5 µL 4× SDS–PAGE loading buffer and 2 µL TCEP to each cell pellet.
9. Heat the samples at 70°C for 10 min prior to SDS–PAGE analysis.
10. Set up a Criterion SDS–PAGE gel of a proper percentage range for the proteins of interest (*see* **Note 30**).
11. Fill the upper and lower chambers of the gel apparatus with 1× TGS buffer.
12. Load 10 µl of sample in each well, along with 2 µl of Benchmark Protein Ladder as a molecular size standard.
13. Hook up the electrodes, and apply 200 V for 55 min.
14. At the end of the run, remove the gel from its cassette and place it in 100 mL of ultrapure water in a plastic tray. Heat for 1 min in a microwave oven at high power, and shake gently at room temperature for 3–5 min (*see* **Note 31**).
15. Pour off the water and add 70 mL of Gel Stain and microwave on high for 30–45 s or until the stain first starts to boil. Shake the gel gently at room temperature for 30 min (*see* **Note 32**).

16. Destain the gel by removing the stain, and washing 3× in ultrapure water. Add 200 mL ultrapure water and microwave for 2 min. Add 2–3 KimWipes to the tray, and gently shake for 15–30 min at room temperature (*see* **Note 33**).

3.4. Notes

1. The use of TEV protease is preferred by many laboratories for several reasons. First, TEV protease is relatively easy to produce in the lab, thus reducing the costs of purchasing more expensive reagents (e.g., thrombin). Second, TEV sites are rarely found in proteins, increasing the specificity of cleavage. Third, TEV is a highly efficient enzyme and has relatively mild optimal cleavage conditions. Finally, the flexibility at the final amino acid position means that often one can make a truly native aminoterminus after cleavage *(7)*.
2. An additional advantage of the Gateway system is that it is trivial to convert any expression vector (commercial or your favorite lab vector) to a Gateway Destination Vector. The Gateway Destination Vector Conversion Kit contains a Gateway cassette with positive and negative selection markers, and this can be ligated into any blunt-ended restriction site in your vector of interest. The positive selection marker (CAT) enables easy selection of the final Destination vector, and there are three different reading frame cassettes available to fit any vector. The whole process takes no more than 2–3 days to complete, and the final vector can be used in a Gateway LR reaction.
3. Adapter PCR with more than two nested adapters tends to be highly inefficient. If more than two adapters are required on the same end of the DNA, we suggest considering an alternative method of generating the construct. If not, a larger amount of PCR product may be necessary in the Gateway BP reaction due to the small amount of PCR product that contains the full-length product with *att* sites.
4. Overlap PCRs and PCRs with multiple adapters tend to create more side-products called primer-dimers. These are small DNA molecules that result from improper annealing of primers, and which can sometimes contain 1 or 2 *attB* sites. Due to their small size, primer-dimers are highly efficient in the BP recombination reaction and may produce false positive Entry clones.
5. It is a common problem for Gateway cloners to confuse the *attB2* reading frame due to the use of the reverse complement

sequence and its strong similarity to the *attB*1 sequence. We strongly recommend some kind of in silico modeling of Gateway reactions prior to primer design. Several commercial molecular biology software programs can carry out virtual Gateway cloning, including VectorNTI (Invitrogen, Carlsbad, CA). Other programs in common use can often be tricked into carrying out Gateway reactions by pretending that Gateway *att* sites are restriction enzymes. In either case, a quick check of the reading frame is always a worthwhile endeavor.

6. Due to the presence of inverted *attL* repeats in the Entry clones, sequencing of small inserts (<500 bp) can be difficult. For this purpose, one can use special blocking oligonucleotides to eliminate the problem *(8)*, or simply sequence with gene-specific primers. The latter is often easier for single protein cloning projects, while the former is more efficient for high-throughput projects.
7. The addition of 3% DMSO to the PCR can help to minimize the effects of very GC-rich primers or template. Often it is not required, but we have seen no detrimental effect from including it in most PCRs. If templates are very AT rich, it is suggested that the DMSO be left out.
8. The use of large amounts of template DNA helps to dramatically reduce PCR errors by forcing the use of original template molecules for subsequent PCR cycles rather than PCR products which may contain errors. If limited template is available, this amount can be reduced by 10- to 20-fold, but errors will likely increase.
9. Phusion polymerase has become the standard PCR reagent in our lab due to its robust activity and extremely high fidelity. Other polymerases (KOD, Pfu) can also be used, but we would recommend using only high-fidelity enzymes to limit PCR mutations, particularly with long genes. The suggested conditions are optimized for use on BioRad or Applied Biosystems PCR machines; some optimization may be required if PCR machines with slower ramp times are used.
10. The appearance of a properly sized band on an agarose gel does not guarantee the success of the adapter PCR. In most cases, the extra length added by the adapter will not be long enough to distinguish the full-length product from a product of only the first set of primers. For this reason, if failures are observed in BP reactions after adapter PCR, it may be worthwhile to split the PCR into two separate reactions to ensure that the adapter PCR is actually working.
11. Column purification of PCR products is only successful for products >150 bp in length. For smaller products, a PEG

precipitation can be carried out as detailed in the Gateway product manuals. Failure to purify the PCR products will generally lead to a large amount 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 completely.

12. 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 actual concentration.
13. PDonr-223 is one example of a Donor vector – the *attP*-containing vector which becomes the backbone of the Entry clone after the BP reaction. pDonr-223 is a spectinomycin-resistant version of pDonr-221, the standard Gateway Donor vector. We suggest using pDonr-223 rather than pDonr-221 because the kanamycin resistance marker on many cDNA templates can interfere with the kanamycin selection of pDonr-221. Additional Donor vectors are available from Invitrogen (Carlsbad, CA). Note that Donor vectors must be propagated in *E. coli ccdB* Survival or another strain which is resistant to the *ccdB* toxin.
14. To increase the recombination efficiency of the BP reaction an overnight incubation (not longer than 18 h) at 25°C should be performed. A fivefold increase in colonies can be expected, though in some cases, background will also be increased. Also, longer incubations are recommended for PCR products >5 kB.
15. Failure to Proteinase K treat the BP reaction will result in dramatically reduced colony counts due to the inability of the DNA to transform while coated with Clonase proteins.
16. DH5α is a recommended *E. coli* strain for Gateway reactions. However, it can be substituted with any other *recA endA* strain (such as TOP10 or DH10B) if necessary. Be sure that any strain used does not have the F′ episome as it contains the *ccd*A gene which will detoxify the *ccdB* gene resulting in failure of the negative selection. For good BP 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 BP reaction (such as with a very long gene) – usually the number of colonies obtained with standard chemically competent cells is more than sufficient.

17. For high-throughput screening and plating, a regular petri plate (100 × 15 mm) can be divided into three or four quadrants to plate multiple 50-µL transformation mixtures at the same time. The cells can be spread using plastic L-spreaders or inoculating loops.
18. Many commercial kits are available for generating plasmid DNA from *E. coli*. We prefer the FastPlasmid kit for routine plasmid preps due to its high speed and consistent results. FastPlasmid can only be used for DNA generated in EndA$^+$ hosts, as it does not remove nucleases which could affect downstream processes. A standard alkaline lysis plasmid prep will also work – *see* **Section 3.3.6.2, steps 5–14** for a protocol.
19. There are many commercial kits for high-throughput plasmid preparations, but the Qiagen Turbo Plasmid Prep Kit is fast, cheap, and produces DNA of a reasonable quality for downstream sequencing and subcloning reactions.
20. 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 50–100 ng Entry clone to avoid cotransformation problems. Increases in efficiency, if needed, can be achieved by increasing the amount of Destination vector.
21. Destination vectors can be added as either supercoiled plasmids, or as plasmids that have been linearized within the Gateway cassette. Contrary to the manufacturer's claim, using linearized Destination vector will actually improve LR efficiency 2- to 5-fold. However, due to the high efficiency of the LR reaction, we do not find the extra effort required to prepare and purify linearized Destination vector to be worthwhile.
22. To increase the recombination efficiency of the LR reaction an overnight incubation (not longer than 18 h) at 25°C should be performed. However, LR reactions are generally efficient enough that a 1-h incubation at 30°C produces more than sufficient numbers of colonies, and a reduced reaction time may even be acceptable.
23. Gateway reactions are usually so efficient and accurate that further confirmation of Expression clones is not necessary. However, if desired, the Gateway *attB* sites can be cleaved with the restriction enzyme *BsrGI*, which will cut out your gene of interest (if it has no additional sites) and allow verification of insert size. Alternately, other restriction sites can be employed.
24. For some applications, DNA for transfection into mammalian cells must be generated with very low levels of bacterial

endotoxin. For these purposes, plasmid prep kits specifically designed for endotoxin removal should be used.

25. In a DH10Bac transformation, two types of colonies will be observed – blue colonies are nonrecombinant, while white colonies contain the proper recombinant baculovirus. Some white colonies may contain small amounts of nonrecombinant baculovirus which can affect downstream success. In order to ensure low background, if time is available white colonies should be restreaked onto fresh LB-KGXTI plates and grown for an additional day at 37°C. Colonies which produce streaks with any blue color at all should be discarded. You should also expect to find that the blue colonies are smaller in size – for this reason, the largest colonies should be picked where possible.
26. DH10Bac cells harbor a single copy plasmid containing the entire baculovirus genome. This "bacmid" DNA cannot be purified by many commercially available kits due to its size and low copy number; however, a classical alkaline lysis preparation will produce enough material for subsequent insect cell transfections.
27. Gel analysis of bacmid DNA is difficult due to the size of the bacmid, interference from leftover plasmid DNA, and low concentration. We prefer PCR verification of bacmids using the suggestions in the manufacturer's Bac-to-Bac kit. The utility and process of the PCR verification will vary depending on your choice of vectors.
28. Choice of expression strains is dependent partially on the type of expression vector being used. In most cases, vectors contain the T7 promoter, and thus require a strain that makes the T7 RNA Polymerase. Common choices are BL21(DE3) or its derivatives. A frequent choice in our laboratory is Rosetta(DE3), which is a derivative of BL21 which contains a plasmid that encodes tRNA genes for rare codons often found in mammalian genes.
29. Lower temperature induction can dramatically improve soluble protein expression for many proteins *(9)*. If an 18°C shaker is not available, induction at 25°C or even 30°C often gives improvement of soluble protein expression. A comparison of solubility at 37°C and lower temperatures may be valuable.
30. Criterion gels offer a wide range of polyacrylamide concentrations to optimize separation of proteins of various molecular weights. We prefer the wide-range 4–12 and 10–20% gels for proteins in the 40–150 and 10–80 kDa ranges, respectively. Criterion offers 26-well gels that have high resolution and fast run times; however, other SDS gel systems can be substituted, and voltages and run times may vary by manufacturer.

31. The heating in water is an essential step to remove SDS from the gel prior to staining. Elimination of this step will result in significantly less sensitive staining, as the dye will not bind well to regions of the gel coated with detergent. Contrary to many published protocols, fixation of the gel is not required, and a short soaking in water will not adversely affect resolution of your protein samples.
32. This staining protocol produces the same sensitivity as traditional Coommasie Blue staining without the need for methanol and in considerably less time. It is equivalent to the commercially available "safe" staining reagents such as Invitrogen's SimplyBlue or Pierce's GelCode Blue in sensitivity and speed, but is considerably cheaper.
33. We find that KimWipes are an economical destaining agent, soaking up much of the dye and accelerating the destaining process significantly. One must be careful not to allow gels to destain for too long or protein bands will begin to destain. If time is a problem, gels should be stained as mentioned, and then diluted with 100 mL water and let sit overnight. Destaining can then be carried out the next day. We do not recommend leaving the gels in destain overnight.

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