HHS Public Access

Author manuscript

Methods Enzymol. Author manuscript; available in PMC 2018 July 02.

Published in final edited form as:

Methods Enzymol. 2017 ; 592: 1–26. doi:10.1016/bs.mie.2017.03.008.

MacroBac: New technologies for robust and efficient large-scale production of recombinant multi-protein complexes

S. Gradia[1,6], **J.P. Ishida**[2,7], **Miaw-Sheue Tsai**[3], **C. Jeans**[1], **John A. Tainer**[2,4,8], and **J.O. Fuss**[2,5,8]

[1]QB3 MacroLab, California Institute for Quantitative Biosciences, University of California, Berkeley, CA, United States

[2]Molecular Biophysics and Integrated Bioimaging, Lawrence Berkeley National Laboratory, Berkeley, CA, United States

[3]Biological Systems and Bioengineering, Lawrence Berkeley National Laboratory, Berkeley, CA, United States

[4]The University of Texas M. D. Anderson Cancer Center, Houston, Texas, United States

[5]Cinder Biological, Inc., 820 Heinz Avenue, Berkeley, CA, United States

Abstract

Recombinant expression of large, multi-protein complexes is essential and often rate-limiting for determining structural, biophysical, and biochemical properties of DNA repair, replication, transcription, and other key cellular processes. Baculovirus infected insect cell expression systems are especially well-suited for producing large, human proteins recombinantly and multi-gene baculovirus systems have facilitated studies of multi-protein complexes. In this chapter, we describe a multi-gene baculovirus system called MacroBac that uses a Biobricks-type assembly method based on restriction and ligation (Series 11) or ligation independent cloning (LIC) (Series 438). MacroBac cloning and assembly is efficient and equally well-suited for either single subcloning reactions or high-throughput cloning using 96-well plates and liquid handling robotics. MacroBac vectors are polypromoter with each gene flanked by a strong polyhedrin promoter and an SV40 poly(A) termination signal that minimize gene order expression level effects seen in many polycistronic assemblies. Large assemblies are robustly achievable, and we have successfully assembled as many as 10 genes into a single MacroBac vector. Importantly, we have observed significant increases in expression levels and quality of large, multi-protein complexes using a single, multi-gene, polypromoter virus rather than co-infection with multiple, single-gene viruses. Given the importance of characterizing functional complexes, we believe that MacroBac provides a critical enabling technology that may change the way that structural, biophysical, and biochemical research is done.

[8]Corresponding authors: jfuss@lbl.gov or jatainer@gmail.com.

[6]Current address: Caribou Biosciences, Inc., 2929 7th Street, Suite 105, Berkeley, CA, United States

[7]Current address: BioMarin Pharmaceutical Inc., 105 Digital Drive, Novato, CA, United States

Keywords

Baculovirus; insect cells; MacroBac; multigene; protein complex; recombinant protein expression; Biobricks; LIC

1. Introduction

DNA repair and other processes in the cell rely on large, multi-protein complexes to catalyze reactions, coordinate product intermediate hand-offs, and scaffold functional protein assemblies (Fuss and Tainer, 2011, Hammel et al., 2016, Lafrance-Vanasse et al., 2015, Williams et al., 2014). In order to understand the biochemical and structural properties of these complexes, researchers have either purified complexes from large amounts of native material or produced and purified recombinant versions (Greimann and Lima, 2008, Schultz et al., 2000).

For more than 30 years, baculovirus infected insect cell expression systems have been the method of choice for producing large human proteins or multi-protein complexes recombinantly (Smith et al., 1983). In order to express multiple proteins at once, researchers have typically made separate viruses for each protein of interest and then co-infected cells with multiple viruses at once. Unfortunately, this co-infection method rarely results in multiple viruses simultaneously infecting a single cell. Vijayachandran et al. best demonstrated the limits of co-infection by co-expressing two viruses each containing a different fluorescent protein and quantifying cells positive for both viruses in fluorescent micrographs (Vijayachandran et al., 2011). Even adjusting virus ratios or using a high multiplicity of infection (MOI) for the two viruses resulted in co-infection of less than 50% of cells (Vijayachandran et al., 2011). Although infection of cells by a single virus can be predicted by the Poisson distribution, co-infection frequencies by two viruses cannot be extrapolated (Mena et al., 2007). Given these limitations with just two viruses, probabilities of co-infection of single cells with more than two viruses is likely to be extremely rare. Therefore, production of multi-protein complexes by baculovirus co-infection in bulk cultures results in populations of singly-expressed proteins and co-expressed complexes that mix upon cell lysis. These infection mixtures not only greatly limit expression levels, but also have the potential to cause heterogeneity that would complicate biochemical, biophysical, and/or structural analysis of multi-protein complexes.

Fortunately, baculovirus expression systems are no longer limited to expressing a single gene from a single virus. Several multi-protein baculovirus systems exist to aid researchers in expressing multiple genes from a single virus. One such system is the MultiBac system that was developed over ten years ago and has evolved over several generations (Trowitzsch et al., 2010). MultiBac uses site-specific recombination by the Cre enzyme to accept an array of plasmids that facilitate multigene assembly (Nie et al., 2014, Sari et al., 2016, Thimiri Govinda Raj et al., 2014). Another system is biGBac that uses Gibson assembly of linear DNA fragments flanked by computationally optimized linker sequences (Gibson et al., 2009, Weissmann et al., 2016).

Biobricks™ was introduced by Tom Knight at MIT as an effort to standardize assembly techniques for DNA sequences (Knight, 2003). In a Biobricks design all parts are flanked by identical restriction enzyme sites allowing any part to be combined with another by a single cloning scheme. Two of the restriction sites have compatible overhangs that cannot be cut after ligation resulting in irreversible fusion of two parts. The flanking restriction sites remain intact upon ligation and the fused product can be used in subsequent assembly reactions. Ligation-independent cloning or LIC is an efficient method that relies on annealing 15–25 base single-stranded complementary DNA ends to clone a DNA insert into a plasmid. Sequences of the DNA ends are designed so that when treated with T4 DNA polymerase and a single deoxynucleotide triphosphate, the 3′→5′ exonuclease activity of the polymerase produces discrete 5′ overhangs. A LIC treated DNA insert is annealed to an analogously treated complementary vector and transformed directly into *E. coli* without ligation (Aslanidis and de Jong, 1990). In our experience, LIC is robust and efficient, with success rates >90% even with large constructs, and equally well-suited for either small-scale or high-throughput cloning using 96-well plates and liquid handling robotics.

Here, we describe a novel multigene baculovirus system called MacroBac that uses Biobricks-style assembly with either restriction and ligation (Series 11), or LIC (Series 438) to build polypromoter expression plasmids. The efficiency and robustness of LIC has prompted us to switch to using the Series 438 MacroBac vectors almost exclusively; however, some users may prefer the familiarity of restriction and ligation assembly with the Series 11 vectors, so we describe both here.

MacroBac plasmids are derived from the pFastBac vector that is part of the Bac-to-Bac baculovirus expression system sold by the Invitrogen Life Technologies brand of Thermo Fisher Scientific (Life_Technologies, 2015). Like pFastBac, heterologous genes subcloned into MacroBac are placed under the transcriptional control of the strong polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV) that is expressed during the late stages of infection while an SV40 poly(A) signal provides polyadenylation and termination. MacroBac vectors are polypromoter such that, after assembly, each gene is driven by an upstream polyhedrin promoter and terminated by an SV40 poly(A) signal. We find that the order of assembled genes has little effect on protein expression levels in polypromoter constructs, providing a significant advantage over polycistronic constructs.

2. Entry cloning into MacroBac Series 11 or Series 438 by ligation independent cloning

MacroBac vectors are available with various tags and fusion proteins (Table 1) and can be obtained from either Addgene or the University of California, Berkeley MacroLab for a small fee (please see acknowledgements). Both MacroBac Series 11 and 438 use LIC for initial entry of an open reading frame (ORF) into the vectors. Table 1 lists the LIC tags to be used in PCR primer design for each MacroBac vector. Complementary LIC sequences are found in the Series 11 and 438 vectors on either side of a SspI (or HpaI) restriction site. To add an ORF of interest to these vectors, the vector is restricted and then both the vector and PCR product are treated with T4 DNA polymerase and a single dNTP (Fig. 1A). For Series

11 and 438 (except the SNAP-v3 vector), the vector is treated with dGTP and the PCR product is treated with dCTP. The exonuclease activity of T4 DNA polymerase removes terminal nucleotides in the 3′ to 5′ direction until the exonuclease stalls in the presence of a single nucleotide, creating complementary overhangs of 15 bases (Fig. 1B). The treated vector and PCR product are then annealed and transformed directly into *E. coli*.

The protocols below are written sequentially such that the vector is digested, gel-purified, and LIC treated first and then the PCR insert is digested, gel-purified, and LIC treated. In practice, however, it is more time efficient to digest, gel-purify, and LIC treat both the vector and the insert in parallel. We have also observed increased cloning efficiencies if the LIC reaction is performed immediately before annealing and transforming into *E. coli*.

Solutions and reagents

- Expression vector (Series 11 or 438 obtained from Addgene or MacroLab)
- ORF Template DNA
- SspI (or HpaI) (New England Biolabs catalog #R0132 (or #R0105))
- 10× SspI (or CutSmart) buffer (New England Biolabs, included with enzyme)
- Agarose and TAE buffer
- 6× DNA loading dye
- Macherey-Nagel Gel and PCR clean-up (or similar) kit (Macherey-Nagel catalog #740609)
- dGTP and dCTP (25 mM stock)
- T4 DNA polymerase (LIC–qualified from EMD/Novagen catalog #70099)
- 10× T4 DNA pol buffer
- 100 mM DTT
- PCR primers (with LIC tags and complementary sequence to ORF)
- PCR polymerase (suggest KOD DNA polymerase from EMD Millipore catalog #71085 or 71086)
- 10× PCR pol buffer
- dNTP mix
- XL1-Blue Competent Cells (Agilent Technologies catalog #200249)
- LB agar plates with 50–100 μg/mL ampicillin
- LB
- 2YT broth
- Ampicillin (100 mg/mL stock)
- Qiagen (or similar) Mini-prep Kit

- pFastBac sequencing primers

  pFastBac Forward (20-mer): 5′-GGATTATTCATACCGTCCCA-3′

  pFastBac Reverse (20-mer): 5′-CAAATGTGGTATGGCTGATT-3′

Prepare LIC vector

1. Digest 500 ng of the expression vector with SspI (or HpaI for SNAP-v3 vectors) and run it on an agarose gel being sure to run long enough to separate the linearized band from anything that appears in an uncut control. Co-purification of your linear DNA with supercoiled or nicked circular DNA will result in a high background when cloning.
2. Use Macherey-Nagel kit (or similar) to perform a gel purification of the linearized plasmid. Elute from the filter with 30 μL of elution buffer. The concentration of the eluted DNA is usually between 5–15 ng/μL.
3. Perform LIC reaction on your linearized plasmid:

10 μL	gel purified vector (50–150 ng)
2 μL	dCTP or dGTP (25 mM stock) – see Table 1
2 μL	T4 DNA pol 10× Buffer
1 μL	100 mM DTT
0.4 μL	EMD/Novagen T4 DNA pol
4.6 μL	H_2O

Use a thermocycler to incubate at 22°C for 30 minutes then 75°C for 20 minutes.

Prepare LIC PCR

1. PCR your gene of interest using gene specific primers with added LIC tags.
2. Run 10 μL of a PCR reaction on a 1% agarose gel.
3. Use a Macherey-Nagel kit (or similar) to perform a PCR cleanup reaction on the remaining PCR. It is essential that NO free dNTPs are present in your PCR product. If your PCR yields multiple bands or you are amplifying from a plasmid template with the same antibiotic resistance as your target vector, then it is best to gel purify your PCR product. (A DpnI digest may also be used to eliminate a problematic plasmid template from your PCR).
4. Perform a LIC reaction on your PCR:

10 μL	gel purified PCR product (or 5 μL of PCR cleanup reaction, 50–150 ng DNA)
2 μL	dCTP or dGTP (25 mM stock) – see Table 1
2 μL	T4 DNA Pol 10× Buffer
1 μL	100 mM DTT
0.4 μL	EMD/Novagen T4 DNA pol
4.6 μL	(or 9.6μL) H_2O

Use a thermocycler to incubate at 22°C for 30 minutes then 75°C for 20 minutes.

Anneal LICed vector and LICed PCR product, and transform

1. Combine 2 μL LICed PCR product + 2 μL LICed vector + 6 μL H_2O
2. Incubate 10 min at room temperature to anneal.
3. Transform into chemically competent XL1-Blue or similar cloning strain:

   Incubate for 30 minutes on ice.

   Heat shock at 42°C for 30 seconds.

   Cool on ice for 2 minutes.

   Add 900 μL 2YT and shake at 37°C for 1 hour.

   Plate 150 μL on LB/Amp plate.

   Grow at 37°C overnight.

A no-insert control of your vector should yield no colonies.

Screen colonies by restriction enzyme digestion

1. Select 2 colonies from each plate and grow in 2 mL LB + 50 μg/mL ampicillin overnight.
2. Mini-prep overnight cultures using a Qiagen mini-prep kit.
3. Linearize plasmids with NotI (or appropriate enzyme(s)) as follows:

4 μL	Plasmid
1 μL	10× CutSmart buffer
0.2 μL	NotI
4.8 μL	H_2O

4. Incubate ~1 hour at 37°C.
5. Add 2 μL 6× DNA loading dye and run all on 1% agarose gel with a 1 kb ladder.
6. Select plasmids that yield the correct size restriction digest fragment(s).
7. Verify construct by sequencing with pFastBac forward and reverse primers.

3. Building complexes by restriction and ligation: MacroBac Series 11

Once all genes of interest are subcloned into Series 11 vectors by LIC (Section 2), the genes can be assembled using a Biobricks cloning scheme (Fig. 2A). MacroBac Series 11 vectors are modified pFastBac (Thermo Scientific) vectors. NotI and PacI restriction sites were added 5′ to the polyhedrin promoter and AsiSI and SbfI restriction sites were added 3′ to the SV40 poly(A) signal. In order to use Series 11 vectors, none of your ORFs can contain NotI, PacI, AsiSI, or SbfI restriction sites. Since these enzymes all have 8 base pair recognition sites, their occurrence is rare.

When genes are linked together, the resulting construct is polypromoter, with each gene under the control of its own polyhedrin promoter. In our experience, this minimizes the

effect of gene order on expression levels that is typically seen in polycistronic assemblies. After assembly, the SV40 poly(A) signal and polyhedrin promoter sequences will be found between each gene with a frequency of n-1 (n=number of genes). As assemblies grow, it is possible that these repeats may cause plasmid stability problems in *E. coli*. However, we have found assemblies of even 10 genes are stable when carried in XL1-Blue *E. coli* strains.

The basic Biobricks assembly scheme is shown in Fig. 2A. Our preferred scheme for subcloning is using the AsiSI, PacI, and NotI enzymes. The plasmid carrying Gene 2 acts as a destination vector and is linearized using NotI and PacI, then treated with CIP. Gene 1 is isolated from its plasmid backbone using NotI and AsiSI. Gene 1 is then ligated into the plasmid containing Gene 2. AsiSI and PacI have complementary overhangs but are not recleavable. Therefore, the restriction site configuration after assembly is unchanged (NotI/PacI on 5′ side and AsiSI/SbfI on the 3′ side) and can be used in a subsequent reaction to link more genes together (Fig. 2B).

It is also possible to subclone using the AsiSI, PacI, and SbfI enzymes. The gene order ends up being the reverse of Fig. 2A. We have observed higher efficiency subcloning using the AsiSI, PacI, and NotI method so we suggest using the scheme shown in Fig. 2A.

Although ligation mediated Biobricks cloning is efficient, as the insert and/or destination plasmid exceeds 12 kb efficiency will decrease. Having cloned plasmids up to 25 kb using this method, we have found that as constructs becomes bigger, a molar ratio of plasmid:insert in the ligation should be closer to 1:1 than the usual 1:2.5 and it may become necessary to use super-competent XL1-Blue cells. If large assemblies (>15 kb) are desired, we recommend using Series 438 MacroBac plasmids.

Biobricks-style subcloning allows assemblies to be built in parallel so an 8-gene construct can be assembled in three rounds of subcloning. Four sets of 2-gene constructs are assembled in the first round. Two sets of 4-gene constructs are assembled in the second round. The final 8-gene construct is assembled in the third round.

The protocols below are written sequentially, however, it is more time efficient to prepare both the vector and the insert in parallel.

Solutions and reagents

- Gene 1 and Gene 2 Series 11 MacroBac plasmids (prepared as in Section 2 and mini-prepped with Qiagen mini-prep kit)
- AsiSI, PacI, and NotI restriction enzymes (New England Biolabs catalog #R0630, R0547, and R0189)
- 10× CutSmart buffer (New England Biolabs, included with AsiSI and PacI enzymes)
- Calf Intestinal Alkaline Phosphatase (CIP) (New England Biolabs catalog #M0290)
- Agarose and TAE buffer

- 6× DNA loading dye
- Macherey-Nagel Gel and PCR clean-up (or similar) kit (Macherey-Nagel catalog # 740609)
- 4× Quick ligation buffer
  - 264 mM Tris-HCl pH 7.6
  - 40 mM $MgCl_2$
  - 4 mM DTT
  - 4 mM ATP pH 7.0
  - 24% PEG 6K
- T4 Ligase (New England Biolabs catalog #M0202)
- XL1-Blue Competent Cells (Agilent Technologies catalog #200249)
- LB
- 2YT broth
- LB agar plates with 50–100 μg/mL ampicillin

Linearize the destination vector

1. Set up restriction digests to linearize the destination vector (e.g. Gene 2 MacroBac):

5 μL	Vector (~0.5 μg)
2 μL	10× CutSmart buffer
0.5 μL	NotI
0.5 μL	PacI
12 μL	H_2O

2. Incubate at 37°C for 2.5 hours.
3. CIP treat the destination plasmid by adding 0.5 μL CIP (NEB).
4. Incubate at 37°C for 5 minutes (do not over-CIP treat).
5. Run samples on a 1% agarose gel.
6. Cut out the appropriate bands and gel purify them (with Macherey-Nagel kit or similar). Samples will be at low concentrations (5–15 ng/μL).

Prepare the gene insert

1. Set up restriction digests to cut out the gene insert (e.g. Gene 1 MacroBac):

5 μL	Vector (~0.5 μg)
2 μL	10× CutSmart buffer
0.5 μL	NotI
0.5 μL	AsiSI
12 μL	H_2O

2. Incubate at 37°C for 2.5 hours.
3. Run samples on a 1% agarose gel.
4. Cut out the appropriate bands and gel purify them (with Macherey-Nagel kit or similar). Samples will be at low concentrations (5–15 ng/μL).

Ligate destination vector and gene insert

1. Set up ligation reaction (include a no-insert control):

5 μL	4× Quick-ligation buffer
1 μL	T4 ligase (NEB)
10 μL	insert
4 μL	vector

2. Incubate at room temperature for ~30 minutes.
3. Transform 6 μL into XL1-Blue

   Incubate on ice for 30 minutes.

   Heat shock at 42°C for 30 seconds.

   Cool on ice for 2 minutes.

   Add 900 μL 2YT.

   Shake at 37°C for 1 hour.

   Plate 150 μL on LB/amp plate.

   Grow at 37°C overnight.

Screen colonies with a PacI/XhoI restriction digest

A PacI/XhoI digest can be used to screen colonies for gene assembly. PacI will cut at the beginning of the assembled genes and XhoI will cut between each gene. If your genes contain additional XhoI sites take these into account when analyzing the restriction digest results. Fig. 3 shows an example of PacI/XhoI digest of a Series 11 MacroBac vector containing 7 genes.

1. Select 2 colonies from each plate and grow in 2 mL + 50 μg/mL ampicillin overnight.
2. Mini-prep overnight cultures using a Qiagen mini-prep kit.
3. Digest plasmids with PacI and XhoI

4 μL	Plasmid
1 μL	10× CutSmart buffer
0.2 μL	PacI
0.2 μL	XhoI
4.6 μL	H_2O

4. Incubate ~1 hour at 37°C.

5. Add 2 μL 6× DNA loading dye and run all on 1% agarose gel with a 1 kb ladder.
6. Select plasmids that yield the correct size restriction digest fragments.
7. Verify construct by sequencing with gene specific primers.

4. Building complexes by ligation independent cloning: MacroBac Series 438

The 438 series plasmids are similar to the Series 11 but use LIC to perform the Biobricks-style assembly of polypromoter genes. This LIC method was developed to facilitate assembly of very large constructs, but can be used for any size assembly. LIC subcloning eliminates cloning background due to re-ligation by ligase so cloning efficiency is very high even with large plasmids.

Like Series 11, Series 438 MacroBac uses a pFastBac backbone that has been modified to accommodate a Biobricks-type architecture that allows for repeated assembly of target ORFs. Series 438, however, uses restriction digestion followed by LIC treatment to generate the desired polypromoter expression plasmids. LIC sequences and a PmeI site were inserted 5′ to the polyhedrin promoter and LIC sequences, SwaI, and PmeI sites were added 3′ to the SV40 poly(A) signal. This configuration preserves the advantages of a Biobricks assembly while enabling LIC.

After all of the genes of interest are cloned into Series 438 vectors (Section 2), gene assembly proceeds by digesting the plasmid containing Gene 1 with SwaI (Fig. 4A). The Gene 1 plasmid will act as the destination vector. Gene 2 is isolated from its plasmid backbone by digestion with PmeI. Both destination vector and insert are treated with T4 DNA polymerase and a single nucleotide (dGTP or dCTP), annealed, and transformed into *E. coli*. The complementary overhangs of the insert and destination vector are not the same length so small gaps are present after annealing (Fig. 4B). *E. coli* machinery repairs these gaps in addition to ligating the insert and vector together.

As with the Series 11 plasmids, it is possible to work in parallel so assemblies can go from 2 to 4 to 8 genes in three rounds of subcloning. Four sets of 2-gene constructs are assembled in the first round. Two sets of 4-gene constructs are assembled in the second round. The final 8-gene construct is assembled in the third round.

The protocols below are written sequentially, however, it is more time efficient to digest, gel-purify, and LIC treat both the vector and the insert in parallel.

Solutions and reagents

- Gene 1 and Gene 2 Series 438 MacroBac plasmids (prepared as in Section 2 and mini-prepped with Qiagen mini-prep kit)
- PmeI and SwaI restriction enzymes (New England Biolabs catalog #R0560 and R0604)
- 10× CutSmart buffer (New England Biolabs supplied with PmeI)

- 10× Buffer 3.1 (New England Biolabs supplied with SwaI)
- Agarose and TAE buffer
- 6× DNA loading dye
- Macherey-Nagel Gel and PCR clean-up (or similar) kit (Macherey-Nagel catalog # 740609)
- dGTP and dCTP (25 mM stock)
- T4 DNA polymerase (LIC–qualified from EMD/Novagen catalog #70099)
- 10× T4 DNA pol buffer
- 100 mM DTT
- XL1-Blue Competent Cells (Agilent Technologies catalog #200249)
- LB
- 2YT broth
- LB agar plates with 50–100 μg/mL ampicillin

Prepare the destination vector

1. Set up restriction digests to linearize the destination vector with SwaI (e.g. Gene 1 MacroBac)

5 μL	Vector (~0.5 μg)
2 μL	10× Buffer 3.1 (NEB)
0.5 μL	SwaI
12.5 μL	H_2O

2. Incubate at 25°C for 2.5 hours.
3. Run samples on a 1% agarose gel.
4. Cut out the appropriate bands and gel-purify them (with Macherey-Nagel kit or similar). Samples will be at low concentrations (5–15 ng/μL).
5. Perform LIC reaction on your linearized plasmid:

10 μL	gel purified vector
2 μL	dGTP (25 mM stock)
2 μL	T4 DNA pol 10× Buffer
1 μL	100 mM DTT
0.4 μL	EMD/Novagen T4 DNA pol
4.6 μL	H_2O

Use a thermocycler to incubate at 22°C for 30 minutes then 75°C for 20 minutes.

Prepare the gene insert

1. Set up restriction digest to cut out the gene insert (e.g. Gene 2 MacroBac):

5 μL	Vector (~0.5 μg)

2 μL	10× CutSmart buffer
0.5 μL	PmeI
12.5 μL	H_2O

2. Incubate at 37°C for 2.5 hours.
3. Run samples on a 1% agarose gel.
4. Cut out the appropriate bands and gel-purify them (with Macherey-Nagel kit or similar). Samples will be at low concentrations (5–15 ng/μL).
5. Perform LIC reaction on your excised gene inserts:

10 μL	gel purified gene insert
2 μL	dCTP (25 mM stock)
2 μL	T4 DNA pol 10× Buffer
1 μL	100 mM DTT
0.4 μL	EMD/Novagen T4 DNA pol
4.6 μL	H_2O

Use a thermocycler to incubate at 22°C for 30 minutes then 75°C for 20 minutes.

Anneal LICed vector and LICed insert, and transform

1. Combine 2 μL LICed insert + 2 μL LICed vector + 6 μL H_2O.
2. Incubate 10 min at room temperature to anneal.
3. Transform into chemically competent XL1-Blue or similar cloning strain:

   Incubate for 30 minutes on ice.

   Heat shock at 42°C for 30 seconds.

   Cool on ice for 2 minutes.

   Add 900 μL 2YT and shake at 37°C for 1 hour.

   Plate 150 μL on LB/amp plate.

   Grow at 37°C overnight.

A no-insert control of your vector should yield no colonies.

Screen colonies with a PmeI/XhoI restriction digest

A PmeI/XhoI digest can be used to screen colonies for gene assembly. PmeI will cut at the beginning and end of the assembled genes and XhoI will cut between each gene. If your genes contain additional XhoI sites take these into account when analyzing the restriction digest results.

Select 2 colonies from each plate and grow in 2 mL LB + 50 μg/mL ampicillin overnight.

1. Mini-prep overnight cultures using a Qiagen mini-prep kit.
2. Digest plasmids with PmeI and XhoI:

4 μL	Plasmid
1 μL	10× CutSmart buffer
0.2 μL	PmeI
0.2 μL	XhoI
4.6 μL	H_2O

3. Incubate ~1 hour at 37°C.
4. Add 2 μL 6× DNA loading dye and run all on 1% agarose gel with a 1 kb ladder.
5. Select plasmids that yield the correct size restriction digest fragments.
6. Verify construct by sequencing with gene specific primers.

5. Bacmid preparation and virus production

Like pFastBac, MacroBac constructs need to be transformed into a bacmid-containing *E. coli* strain for site-specific recombination into the viral genome. We recommend using DH10Bac cells (ThermoFisher Scientific) and following instructions for generating recombinant bacmid and baculovirus in the Bac-to-Bac Baculovirus Expression System manual (Life_Technologies, 2015). As the MacroBac construct size increases, it will be necessary to use more DNA than the 1 ng called for in the Bac-to-Bac manual. For constructs over 15 kb, we recommend 5 ng of DNA, but it may be necessary to try several transformations with various amounts of plasmid from 1 to 50 ng. We also recommend increasing the amount of Bluo-gal used in the Luria agar plates to 300 μg/ml to facilitate differentiating blue and white colonies. We recommend restreaking 8 white colonies and preparing bacmid from 4 verified white colonies.

Successful bacmid preparation can be verified by either PCR as described in current versions of the Bac-to-Bac manual or by agarose gel electrophoresis, which we prefer. This method is no longer described in the current Bac-to-Bac manual so we describe it below.

For virus production, we again recommend following the Bac-to-Bac manual for transfecting insect cells and isolating the P1 viral stock (Life_Technologies, 2015).

Visualizing bacmid preparation by agarose gel electrophoresis

1. Pour a 0.5% agarose gel containing 0.5 μg/mL ethidium bromide in TAE buffer.
2. Load 5 μL of the bacmid mini-prep mixed with 1 μL 6× DNA loading dye.
3. Load λ DNA-HindIII Digest (NEB) as a marker following manufacturer's directions.
4. Run gel for 12 hours at 23 V.
5. Image gel.

There should be 4 bands visible (Fig. 5). The bacmid is just above the 23,130 bp band of the λ DNA-HindIII digest. The two lower bands are helper DNA and a much higher molecular weight band is also visible.

6. Expression and stock maintenance

We recommend using cryo-preserved Baculovirus-Infected Sf9 Insect Cells (BIIC) instead of refrigerated baculovirus stocks for large-scale expression of protein complexes (Wasilko et al., 2009). This method can be easily scaled and the frozen stocks are stable for months or years. Briefly, P1 viral stock (the cell supernatant collected after bacmid transfection and 5 days of growth) is used to infect Sf9 cells at a ratio of 1:100 (e.g. 500 μL P1 to 50 mL 2 × 10^6 cells/mL Sf9 cells). After 24 hours, 50 mL of infected cells are spun and resuspended in 10 mL cryo solution (8 mL media, 1 mL 100 mg/mL BSA in media, and 1 mL DMSO). Tubes of 1 mL aliquots are frozen in a cell-freezing container to allow slow freezing over 24 hours at −80°C. After 24 hours, the tubes can be transferred to liquid nitrogen for long-term storage. For protein expression, a 1 mL BIIC stock is thawed, diluted with 5 mL of prewarmed media, and used to infect 5–10 L of culture (we recommend starting with a ratio of 1 mL diluted BIIC stock to 1 L of Sf9 culture). Infected cells are harvested after 4 days by centrifugation, washed with PBS, and either processed immediately or stored as frozen cell pellets at −80°C.

7. Trouble shooting

Paramount to success of the MacroBac system to express target proteins is the use of best cloning practices. Assuring restriction digests are complete, gels are run using methods that yield clearly identifiable bands, and control ligations are performed to ensure low background is obtained will save a tremendous amount of time. Ultimately, the success of a multigene expression bacmid depends on the accuracy of all preceding plasmids, therefore every plasmid should be verified by both sequencing and restriction digest. We recommend using the Series 438 plasmids that use LIC to assemble multiple polypromoter genes. LIC is very efficient and yields very low background levels. This technique is particularly beneficial when working with plasmids >15 kb in size.

When preparing an insert (either by NotI/AsiSI or PacI/SbfI digest) using Series 11 plasmids it is necessary to consider the size of the insert as compared to the vector backbone it was excised from. Ideally the target insert will be clearly separable from the vector backbone it came from so the insert can be gel purified. In cases where a plasmid digest yields an insert and backbone of similar lengths it is best to use this plasmid as a receiving vector rather than an insert. The Series 438 plasmid is beneficial in this scenario; preparation of an insert by a PmeI digest followed by T4 DNA polymerase/dCTP treatment does not result in a vector backbone that can interfere with cloning into a SwaI/T4 DNA polymerase/dGTP prepared plasmid. In theory, it should not be necessary to gel purify either the insert or destination vector when assembling multiple genes in the Series 438 plasmid, however in practice we prefer to visualize the digest and gel purify the desired linearized DNAs from any uncut plasmid that would result in cloning background.

These protocols have been optimized for T4 DNA polymerase purchased from EMD/Novagen. It is possible to use T4 DNA polymerases from other suppliers, but it may be necessary to optimize the protocol using that manufacturer’s instructions as a starting point. For example, some LIC protocols call for a short incubation with EDTA before heating at

75°C for 20 minutes. It may also be necessary to vary the amount of DNA used for LIC annealing, plate more of the recovered cells after transformation, or to screen more colonies to increase LIC efficiencies.

We have not observed instability of the polypromoter MacroBac plasmids in XL1-Blue in spite of the fact that both an identical polyhedrin promoter and SV40 poly(A) termination signal are repeated with each gene. The use of a competent cell line engineered to reduce the frequency of homologous recombination such as Stbl3 (ThermoFisher Scientific) is likely to further decrease the risk of plasmid instability.

8. Future considerations

Biology is written at the level of sequences, but read in the context of dynamic molecular assemblies that have been challenging to prepare in suitable qualities and amounts for characterizations. Biophysical and structural characterizations of large assemblies continue to advance with methods such as small angle X-ray scattering with advanced synchrotron facilities and detectors and cryo-electron microscopy with advanced direct electron detectors (Classen et al., 2013, He et al., 2016, Hura et al., 2013, Rambo and Tainer, 2013). The rate-limiting step is increasingly the ability to efficiently prepare robustly stoichiometric complexes. We therefore anticipate that MacroBac may help address bottlenecks in current structural, biochemical, and biophysical characterizations of large protein complexes and enable testing of structurally-informed mutations and chemical inhibitors in the context of their functionally relevant biological complexes. Such research will provide a mechanistic framework that will allow researchers to not only understand but predict outcomes from disease mutations or designed therapies, thus complementing advances in sequence and systems-level studies.

Acknowledgments

Both MacroBac systems were developed in collaboration with the QB3 MacroLab, a core facility at the University of California, Berkeley that is administered by the California Institute for Quantitative Biosciences (QB3). C.J. is the current MacroLab Director. More information about MacroBac and other MacroLab vectors can be found at http://qb3.berkeley.edu/macrolab/. MacroBac vectors are available from Addgene.org or the MacroLab for a small fee. J.A.T., J.O.F., and M.-S. T. are supported by SBDR P01 CA092584. J.A.T. also acknowledges support of a Robert A. Welch Chemistry Chair, the Cancer Prevention and Research Institute of Texas, and the University of Texas System Science and Technology Acquisition and Retention STARs program.

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Figure 1A

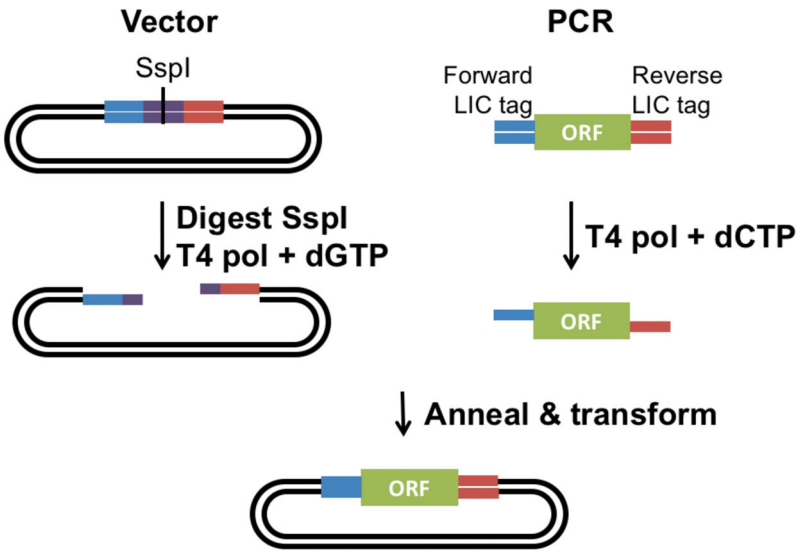

Figure 1B

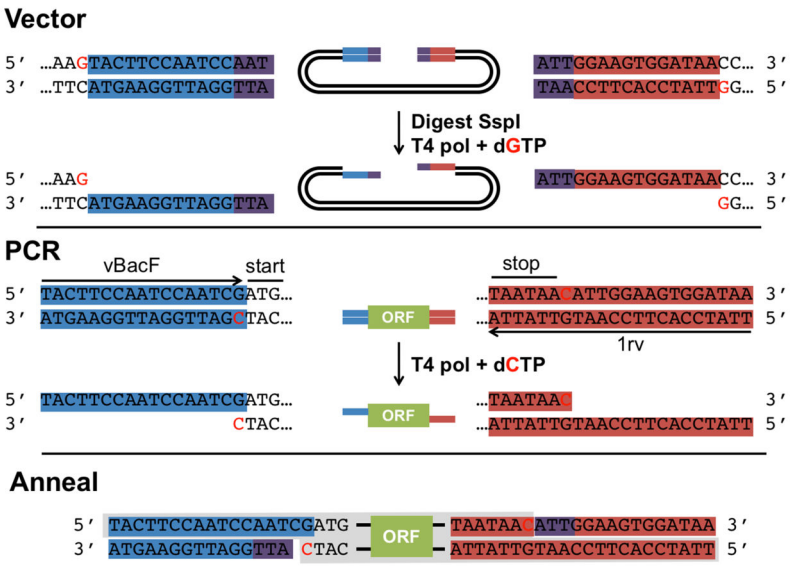

Fig. 1.

(A) Overview of LIC cloning into Series 11 and Series 438 MacroBac vectors. (B) Detailed sequence view of LIC cloning into Series 11A or Series 438-A vectors.

Figure 2A

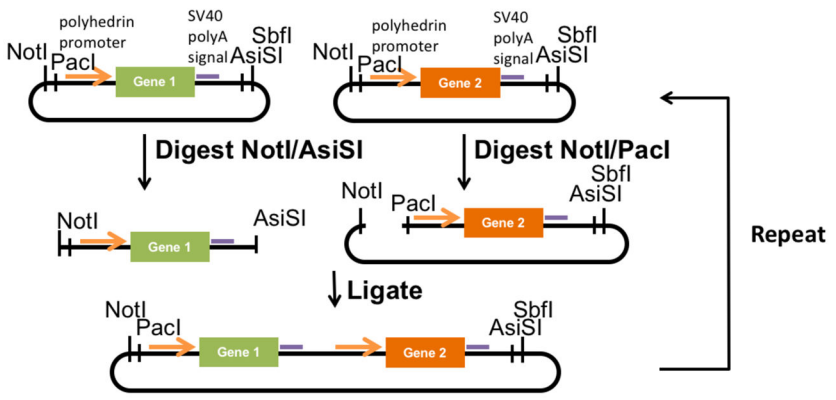

Figure 2B

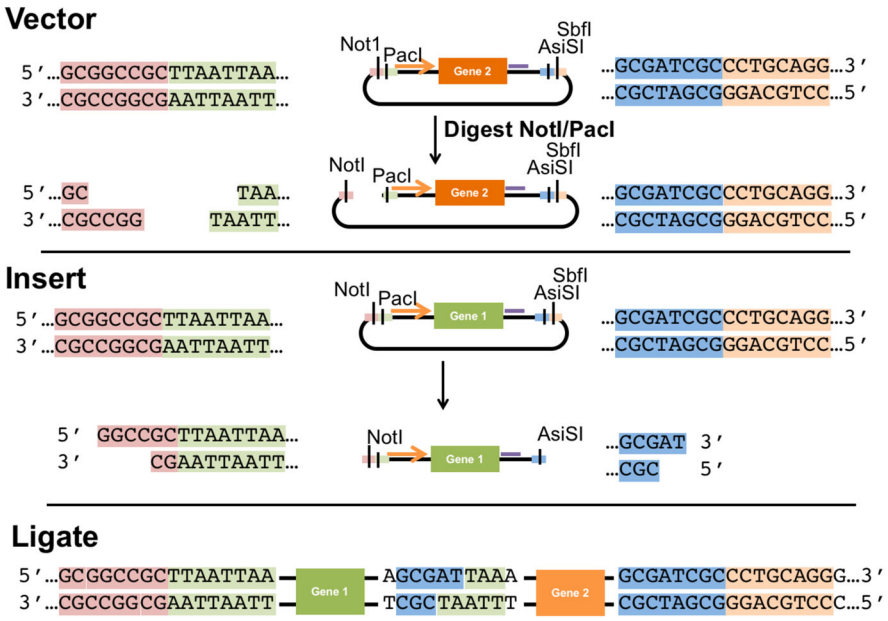

Fig. 2.

(A) Overview of Biobricks restriction and ligation assembly of genes in Series 11 MacroBac. (B) Detailed sequence view of assembly in Series 11 MacroBac.

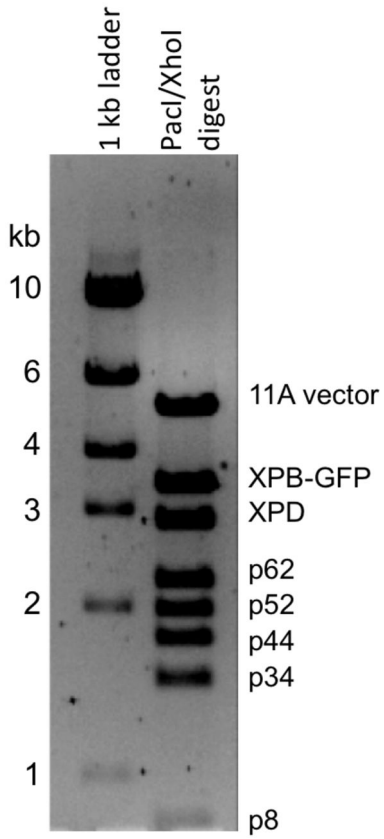

Fig. 3.
PacI/XhoI digest of a Series 11A vector containing 7 genes of human core TFIIH.

Figure 4A

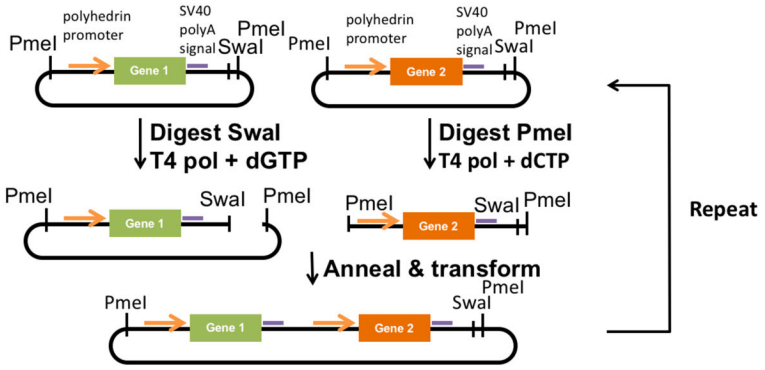

Figure 4B

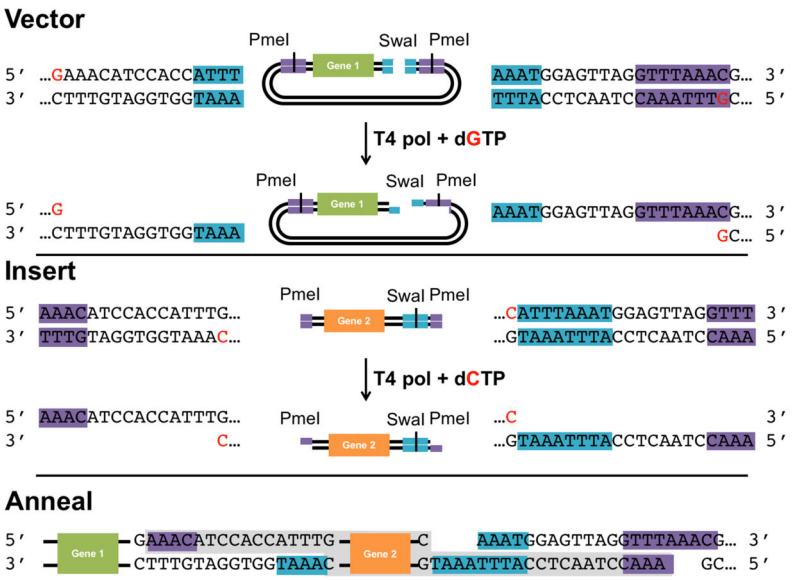

Fig. 4.

(A) Overview of Biobricks-style LIC assembly of genes in Series 438 MacroBac. (B) Detailed sequence view LIC assembly in Series 438 MacroBac.

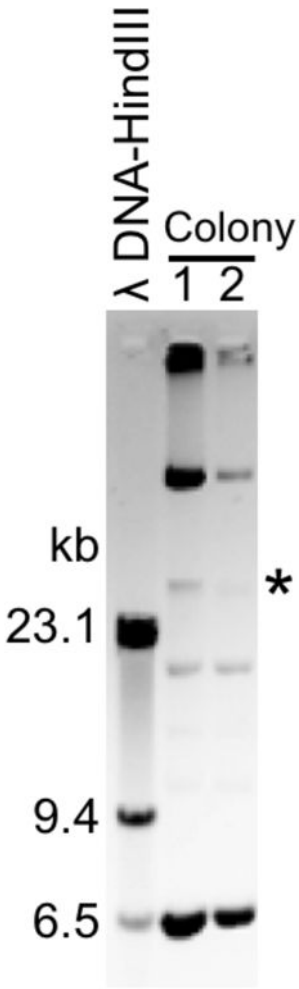

Fig. 5.

Visualization of bacmid preparation by agarose gel electrophoresis. Colony 1 is an example of a successful bacmid preparation with a band just above the 23.1 kb marker (*).

Table 1

MacroBac Series 11 and 438 vectors. Many different tags, protease cleavage, and orientation options are available. All MacroBac vectors are available from Addgene.org and confer ampicillin resistance. The forward and reverse LIC tags should be added to gene specific sequences of the desired ORF to create PCR primers used in Section 2. The restriction sites and dNTPs listed are for LIC entry into the vectors as described in Section 2. Some of the vectors require that the ORF has a starting ATG while others do not. Abbreviations: (yORF) your open reading frame, (His6) 6 histidine tag, (tev) TEV protease cleavage site, (MBP) maltose binding protein, (N10) 10 asparagine linker sequence that may help avoid steric clashes between the protein and MBP tag, (StrepII) streptavidin tag, (msfGFP) superfolder green fluorescent protein, (prescission) PreScission protease cleavage site, (SNAP) SNAP-tag.

Series-11: Baculovirus Expression Vectors - Biobrick Restriction and Ligation

Vector	Addgene #	Tag	Resistance	Forward LIC tag	Reverse LIC tag	Restriction site	Plasmid dNTP	PCR dNTP	Notes
11A	48294	yORF	AMP	(vBac F) TACTTCCAATCCAATCG	(1rv) TTATCCACTTCCAATGTTATTA	SspI	dGTP	dCTP	ORF needs ATG
11B	48295	His6-tev-yORF	AMP	(v1 F) TACTTCCAATCCAATGCA	(1rv) TTATCCACTTCCAATGTTATTA	SspI	dGTP	dCTP	
11C	48296	His6-MBP-N10-tev-yORF	AMP	(v1 F) TACTTCCAATCCAATGCA	(1rv) TTATCCACTTCCAATGTTATTA	SspI	dGTP	dCTP	
11R-GFP	48297	StrepII-msfGFP-tev-yORF	AMP	(v1 F) TACTTCCAATCCAATGCA	(1rv) TTATCCACTTCCAATGTTATTA	SspI	dGTP	dCTP	
11-SNAP-V1	55216	TwinStrepII-prescission-SNAPf-tev-yORF	AMP	(v1 F) TACTTCCAATCCAATGCA	(1rv) TTATCCACTTCCAATGTTATTA	SspI	dGTP	dCTP	
11-SNAP-V3	55217	yORF-tev-SNAPf-prescission-TwinStrepII	AMP	(v3 F) TTTAAGAAGGAGATATAGTTC	(v3 rv) GGATTGGAAGTAGAGGTTCTC	HpaI	dCTP	dGTP	ORF needs ATG

Series-438: Baculovirus Expression Vectors - LIC Subcloning

Vector	Addgene #	Tag	Resistance	Forward LIC tag	Reverse LIC tag	Restriction site	Plasmid dNTP	PCR dNTP	Notes
438-A	55218	yORF	AMP	(vBac F) TACTTCCAATCCAATCG	(1rv) TTATCCACTTCCAATGTTATTA	SspI	dGTP	dCTP	ORF needs ATG
438-B	55219	His6-tev-yORF	AMP	(v1 F) TACTTCCAATCCAATGCA	(1rv) TTATCCACTTCCAATGTTATTA	SspI	dGTP	dCTP	
438-C	55220	His6-MBP-N10-tev-yORF	AMP	(v1 F) TACTTCCAATCCAATGCA	(1rv) TTATCCACTTCCAATGTTATTA	SspI	dGTP	dCTP	
438-Rgfp	55221	StrepII-msfGFP-tev-yORF	AMP	(v1 F) TACTTCCAATCCAATGCA	(1rv) TTATCCACTTCCAATGTTATTA	SspI	dGTP	dCTP	
438-SNAP-v1	55222	TwinStrepII-prescission-SNAPf-tev-yORF	AMP	(v1 F) TACTTCCAATCCAATGCA	(1rv) TTATCCACTTCCAATGTTATTA	SspI	dGTP	dCTP	
438-SNAP-v3	55223	yORF-tev-SNAPf-prescission-TwinStrepII	AMP	(v3 F) TTTAAGAAGGAGATATAGTTC	(v3 rv) GGATTGGAAGTAGAGGTTCTC	HpaI	dCTP	dGTP	ORF needs ATG