Protein complex expression by using multigene baculoviral vectors

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Elucidation of the molecular basis of protein-interaction networks, in particular in higher eukaryotes, is hampered by insufficient quantities of endogenous multiprotein complexes. Present recombinant expression methods often require considerable investment in both labor and materials before multiprotein expression, and after expression and biochemical analysis these methods do not provide flexibility for expressing an altered multiprotein complex. To meet these demands, we have recently introduced MultiBac, a modular baculovirus-based system specifically designed for eukaryotic multiprotein expression[1]. Here we describe new transfer vectors and a combination of DNA recombination–based methods, which further facilitate the generation of multigene cassettes for protein coexpression (Fig. 1), thus providing a flexible platform for generation of protein expression vectors and their rapid regeneration for revised expression studies. Genes encoding components of a multiprotein complex are inserted into a suite of compatible transfer vectors by homologous recombination. These progenitor constructs are then rapidly joined in the desired combination by Cre-*loxP*–mediated *in vitro* plasmid fusion. Protocols for integration of the resulting multigene expression cassettes into the MultiBac baculoviral genome are provided that rely on Tn7 transposition and/or Cre-*loxP* reaction carried out *in vivo* in *Escherichia coli* cells tailored for this purpose. Detailed guidelines for multigene virus generation and amplification, cell culture maintenance and protein production are provided, together with data illustrating the simplicity and remarkable robustness of the present method for multiprotein expression using a composite MultiBac baculoviral vector.

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MATERIALS

REAGENTS

BD In-Fusion recombinase and buffers, (BD Bioscience)
Cre recombinase and 10× reaction buffer (NEB)
Phusion high-fidelity DNA polymerase, 5× HF or 5× GC buffer (Finnzymes or NEB)
PCR primers (see Steps 3 and 4 for details)
*Avr*II, *Pme*I, *Spe*I (NEB), *Bsp*68I (Fermentas) and *Dpn*I (NEB)
QIAprep Spin Miniprep kit, QIAprep Gel Extraction kit, QIAprep PCR purification kit (Qiagen)
T4 DNA ligase, 10× T4 DNA ligase buffer (NEB)
2×TY medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl)
TYE agar plates (1.0% tryptone, 0.5% yeast extract, 0.8% NaCl, 1.5% agar)
Low-salt TYE medium (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl) and agar plates (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar)
Sf-900 II SFM serum free medium (Gibco BRL)
Antibiotics (see **Table 1**)
Isopropylthiogalactoside (IPTG; 1M)
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)
L-arabinose (Gibco BRL)
CellFECTIN (Invitrogen) or FuGENE (Roche)
Template DNA (genomic, plasmid or cDNA)
E. coli competent cells: TOP10, BW23473, DH10MultiBac
Spodoptera frugiperda SF21 cells
Vector DNA: pUCDM, pSPL, pFL, pKL, pFBDM, pKDM
Additional DNA constructs: pBADZHisCre, pUCDM-YFP

EQUIPMENT

Thermocycler programmed with the desired protocols
Equipment for agarose gel electrophoresis and SDS-PAGE
Shaker incubator (temperature-controlled, 27 °C)
Erlenmeyer flasks (500 ml and 2 l)
Fluorescence spectrophotometer and cuvettes (optional)
Electroporator and cuvettes
Sterile hood with UV illumination
6-well (35-mm diameter) tissue-culture plates
4.5 ml CryoTube vials (Nunc)
15 ml and 50 ml Falcon tubes

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Multigene expression design and preparation of target DNAs

PROCEDURE

1| Compile the DNA sequences encoding the components of the multiprotein complex of choice, and plan how you will combine these genes into transfer vectors according to the schematics in **Figures 1** and **2**.

2| All transfer vectors contain a multiplication module that can be used for combining expression cassettes (**Fig. 2**). If you decide to use the multiplication module, make sure the restriction enzymes used for multiplication are not present in the genes you will be cloning.
▲ CRITICAL STEP

3| To insert DNA into the transfer vector of choice by seamless cloning using BD In-Fusion recombinase[2], design PCR primers that contain 20-30 bases annealing to the 5′ and 3′ end of the gene of interest. Add a nonannealing 15–20-base 5′-tail to the primers containing DNA sequences that are homologous to the sequences flanking the site of insertion into the transfer vector to facilitate In-Fusion recombination[2].
Alternatively, insert encoding DNAs into transfer vectors by conventional cloning: amplify DNA fragments using PCR primers containing appropriate restriction enzyme sites for the chosen vector and 18–25 nucleotides complementary to the DNA sequence to be cloned. For both conventional and seamless cloning, if desired, incorporate nucleotide sequences encoding for C- or N-terminal affinity tags into the primers.

4| For vector linearization by PCR, use primers annealing to the sequences flanking the site of insertion (complementary to the 5′ tails of PCR primers in Step 3).
The plasmid used as vector also can be efficiently linearized by restriction digestion, optionally followed by gel extraction. Then nucleotide overhangs present at the restriction site have to be taken into consideration in primer design for the BD In-Fusion reaction as detailed by the manufacturer. In our hands, plasmid linearization by both methods (PCR amplification or restriction digestion) yield comparable results.

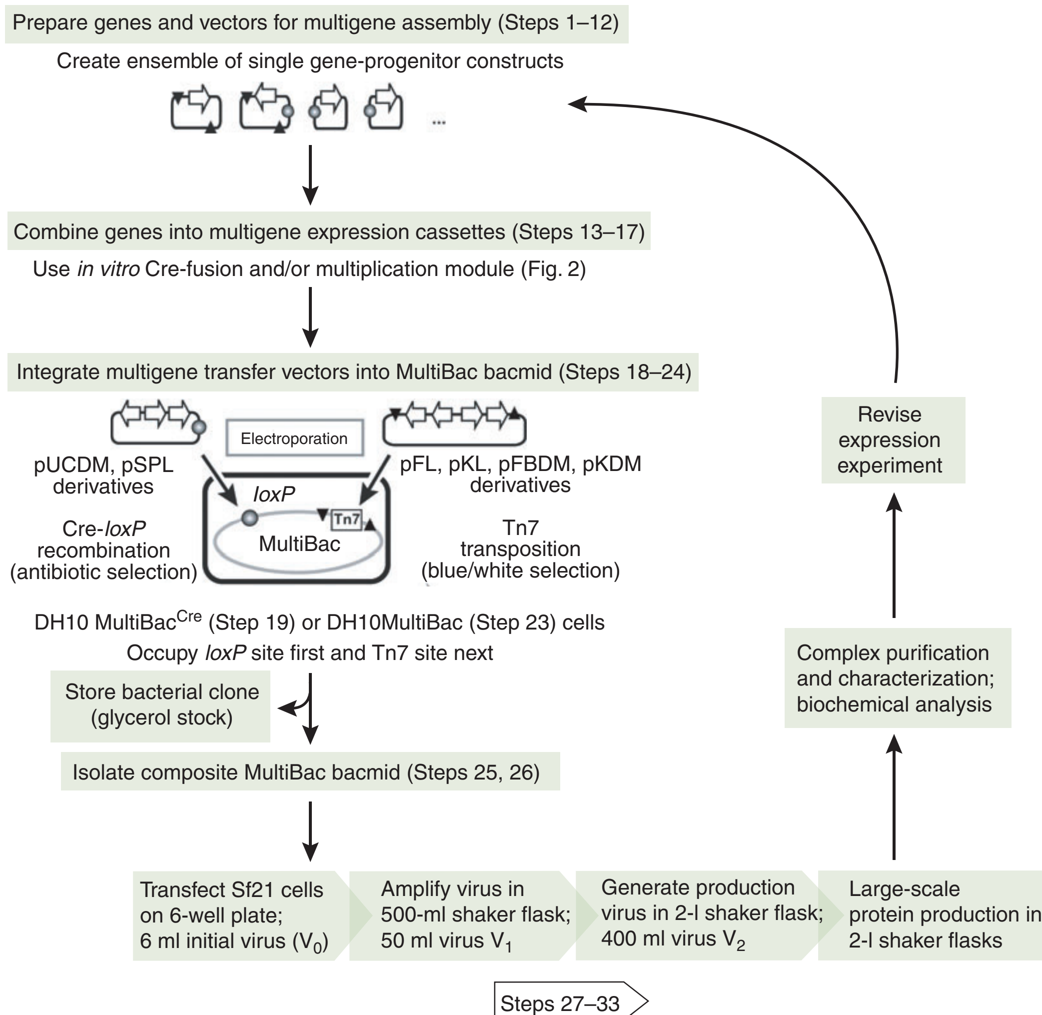

Figure 1 | Outline of the method.

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5| Set up a 50-µl reaction for each DNA insert to be cloned and each vector to be linearized by PCR.

ddH_2O	29 µl
5× Phusion HF Reaction buffer	10 µl
dNTPs (2.5 mM stock)	5 µl
Template DNA (100 ng/µl)	0.5 µl
5′ primer (10 µM stock)	2.5 µl
3′ primer (10 µM stock)	2.5 µl
Phusion polymerase (2 U/µl)	0.5 µl

6| Amplify nucleic acid fragments using the following PCR program:

Cycle number	Denaturation	Annealing	Polymerization	Final
1	2 min at 98 °C	30 s at 65 °C	2 min/kb at 72 °C	
2–30	30 s at 98 °C	30 s at 58 °C (decrease by 0.5 °C/cycle)	1 min/kb at 72 °C	
31			10 min at 72 °C	
Hold				4 °C

➨ **TROUBLESHOOTING**

7| Digest PCR products for 4 h at 37 °C with 20 U of *Dpn*I enzyme. *Dpn*I can be added directly to the 50-µl PCR. Supplement the reaction with 5 µl of 10× NEB buffer 4.
➨ **TROUBLESHOOTING**
▲ **CRITICAL STEP**

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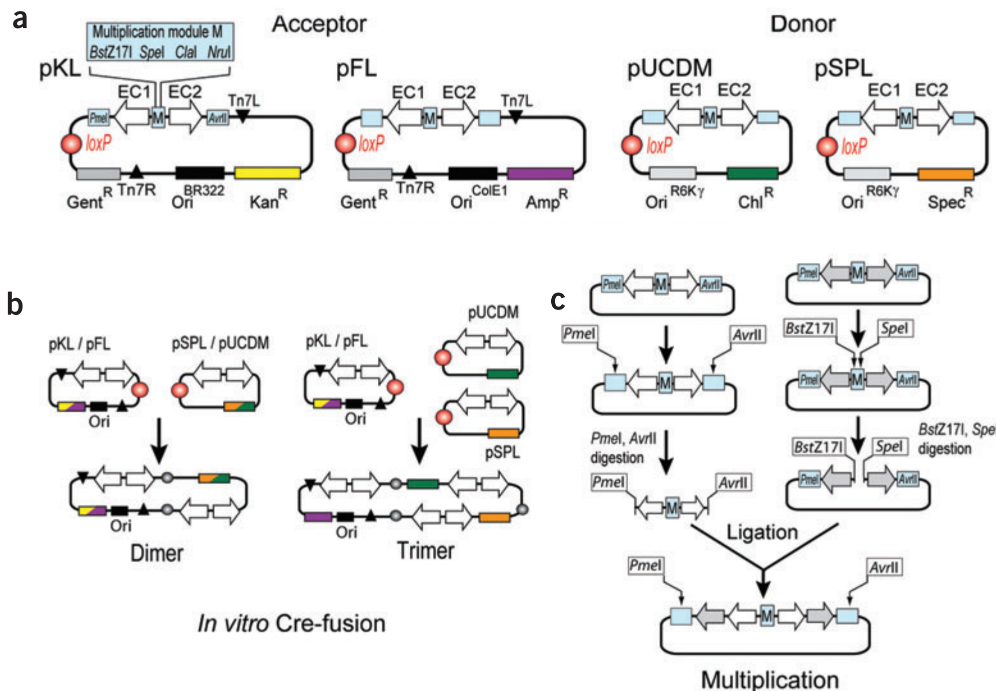

Figure 2 | Creating multigene transfer vectors. (**a**) Vector maps (open arrows are expression cassettes; see also **Supplementary Fig. 1**). (**b**) Multigene transfer vector assembly by *in vitro* fusion of acceptor (pFL, pKL) and donor (pUCDM, pSPL) plasmid derivatives by using Cre recombinase. Acceptor and donor plasmids all contain the *loxP* imperfect inverted repeat (red circles). Derivatives of one acceptor plasmid can be fused to one or two donor plasmids creating multigene plasmid dimers or trimers, respectively. Expression cassettes in between the Tn7 transposition sequences (black triangles) are integrated into the MultiBac baculovirus genome. (**c**) Multigene assembly by using the multiplication module M present on all transfer vectors including also pFBDM and pKDM (**Supplementary Fig. 1**) following the procedure described[1]. Both approaches (*in vitro* plasmid fusion and multiplication via M) can also be used in conjunction for maximal flexibility in multigene vector assembly.

8| If PCR products appear to be sufficiently specific by analytical agarose gel electrophoresis, they can be used directly (Step 9) following purification by QIAprep PCR purification kit. Alternatively, gel purify the desired fragment using the QIAprep Gel Extraction kit.

Cloning of target DNAs into the desired vector

9| Set up BD In-Fusion reaction in 10 μl volume each:

ddH_2O	3.5 μl
10× BD In-Fusion Reaction Buffer	1 μl
10× BSA	1 μl
PCR-amplified insert (200 ng/kb)	2 μl
Linearized vector (100 ng/kb)	2 μl
BD In-Fusion enzyme (diluted)	0.5 μl

The BD In-Fusion enzyme stock concentration is 10×; dilute to 1× with BD In-Fusion dilution buffer and add 0.5 μl of this dilution to the reaction.

10| Incubate the reactions at 37 °C for 30 min and then at 50 °C for 20 min.

11| Use 1–5 μl of the BD In-Fusion reactions to transform chemically competent *E. coli* cells (TOP10, BW23474) and select for growth by plating on TYE agar plates containing the appropriate antibiotics for selection (determined by the vector of choice; see **Table 1**). Incubate overnight at 37 °C.
▲ CRITICAL STEP
■ PAUSE POINT Plates can be stored for several weeks at 4 °C, and completed PCR and In-Fusion reactions can be stored indefinitely at –20 °C.

12| Pick several clones, and isolate plasmid DNA using the QIAprep Spin Miniprep kit. Verify clones for each target by restriction digestion, analytical PCR using the primers designed in Step 4 and/or by sequencing of PCR product or plasmid DNA. Promoter and terminator elements can also be sequenced if vector linearization by PCR was performed.
➨ TROUBLESHOOTING
▲ CRITICAL STEP

13| If both expression cassettes of a transfer vector are to contain an inserted gene each, repeat Steps 4–12 with a clone verified as in Step 12. Alternatively, if PCR steps are to be avoided, follow Steps 4–11 to generate constructs that contain one gene in one of the two expression cassettes of a transfer vector of choice and generate the dual expression vectors via the multiplication module and/or further convenient restriction sites present on the vector backbone.

Table 1 | Features of MultiBac vectors

Vector	Antibiotic	Replicon (source)	Host strain	Recombination and multiplication elements	Usage
pFBDM	Ampicillin Gentamycin	ColE1	TOP10[a]	Tn7L, Tn7R, multiplication module M	Integration in MultiBac Tn7 site
pFL	Ampicillin Gentamycin	ColE1	TOP10	Tn7L, Tn7R, *loxP*, multiplication module M	Acceptor for plasmid fusions; integration in MultiBac Tn7 site
pKDM	Kanamycin Gentamycin	pBR322	TOP10	Tn7L, Tn7R, multiplication module M	Integration in MultiBac Tn7 site
pKL	Kanamycin Gentamycin	pBR322	TOP10	Tn7L, Tn7R, *loxP*, multiplication module M	Acceptor for plasmid fusions; integration in MultiBac Tn7 site
pUCDM	Chloramphenicol	R6Kγ	BW23473	*loxP*, multiplication module M	Integration in MultiBac *loxP* site; donor for plasmid fusions
pSPL	Spectinomycin	R6Kγ	BW23473	*loxP*, multiplication module M	Integration in MultiBac *loxP* site; donor for plasmid fusions
MultiBac bacmid	Kanamycin Ampicillin	F1	DH10MultiBac, DH10MultiBac^{Cre}	Tn7L, Tn7R, *loxP*	Baculovirus receives Tn7 and *loxP* integrands
pBADZ-HisCre	Zeomycin (Zeocin)	ColE1	TOP10, DH10MultiBac^{Cre}	None	Cre expression in DH10MultiBac^{Cre} cells

[a]You may also use any other general laboratory cloning strain ($recA^-$ $endA^-$ pir^-).

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Creating multigene expression cassettes

14| Design multigene expression cassette combinations following the schematic in **Figure 2**. Plasmids pSPL and pUCDM and their derivatives containing expression cassettes are called 'donor plamids' (**Fig. 2** and **Supplementary Fig. 1**). They contain a conditional origin of replication and a *loxP* imperfect inverted repeat. Plasmids pFL and pKL and their derivatives are called 'acceptor plasmids' (**Fig. 2** and **Supplementary Fig. 1**). Acceptor plasmids contain elements of the Tn7 transposon in addition to the *loxP* sequence.
*Donor plasmids containing expression cassettes can be fused via loxP to acceptor plasmids containing further expression cassettes by in vitro Cre-Fusion (**Fig. 2b**). The resulting donor-acceptor fusions can then be integrated into the MultiBac bacmid by Tn7 transposition. Donor plasmids can also be used directly for integration into the MultiBac bacmid by in vivo Cre-loxP reaction (Step 18). Alternatively, multigene expression cassettes can also be generated by using the multiplication module present on all vectors (**Fig. 2c** and **Supplementary Methods**) as described previously[1]. Both approaches can also be used in combination.*

15| Set up plasmid fusion reactions in 10-µl volume each.

ddH_2O	2 µl
10× Cre reaction buffer (NEB)	1 µl
Donor plasmid A (~500 ng)	2 µl
Donor plasmid B (~500 ng)	2 µl
Acceptor plasmid (~500 ng)	2 µl
Cre recombinase	1 µl

If only one donor is fused to the acceptor, add 2 µl of ddH_2O instead of donor plasmid B.

16| Use 1–5 µl of the Cre recombinase reaction products to transform competent *E. coli* cells (for example, TOP10) and select for growth by plating on TYE agar plates containing the appropriate resistance marker combinations (**Table 2**). Incubate overnight at 37 °C.
➨ TROUBLESHOOTING
▲ CRITICAL STEP

17| Pick several clones, isolate plasmid DNA using the QIAprep Spin Miniprep kit and verify clones for each target by restriction digestion and/or sequencing.
▲ CRITICAL STEP
■ PAUSE POINT Plasmids can be stored indefinitely at –20 °C.

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Preparing the composite multigene bacmid

18| Design a strategy to integrate multigene expression cassettes into the *loxP* site and/or the Tn7 site of the MultiBac bacmid (**Fig. 1**). If it is desired to occupy the *loxP* site with donors, this step must be carried out before Tn7 site occupation by fused donor-acceptors.
▲ CRITICAL STEP

Cre-*loxP*–dependent integration of donor derivatives into the MultiBac bacmid

19| Prepare $DH10MultiBac^{Cre}$ cells by transforming the plasmid pBADZHisCre into competent DH10MultiBac cells and selecting for growth on low salt TYE agar plates containing the appropriate antibiotics (**Supplementary Methods**), IPTG and X-gal for color selection. Incubate for >24 h at 37 °C with plates wrapped in parafilm to avoid dehydration.
For integration of acceptor plasmid derivatives or fused donor-acceptors via Tn7 transposition, use DH10MultiBac cells instead (proceed to Steps 24–25).
▲ CRITICAL STEP

20| Pick a single blue colony and prepare electrocompetent $DH10MultiBac^{Cre}$ cells that contain Cre recombinase expressed as a result of L-arabinose induction (see **Supplementary Methods**).
➨ TROUBLESHOOTING
▲ CRITICAL STEP

21| Incubate 10 ng of verified donor derivative for 15 min on ice with 50–100 µl electrocompetent $DH10MultiBac^{Cre}$ cells. After electroporation (200 Ω, 25 µF, 2.0 kV pulse), incubate cells for 8 h or overnight at 37 °C and plate on TYE agar containing the selective antibiotics (**Table 2**), IPTG and X-gal.

22| After 20–30 h incubation at 37 °C, blue colonies should appear. Inoculate blue colonies in appropriate antibiotics and proceed to bacmid preparation for insect-cell infection (Step 25) or prepare electrocompetent cells from clones for integration of an acceptor derivative by Tn7 transposition (**Fig. 1**).
➨ TROUBLESHOOTING
▲ CRITICAL STEP

Tn7 transposition of acceptor derivatives into the MultiBac bacmid

23| Incubate ~10 ng of verified acceptor derivative for 15 min on ice with 50–100 µl electrocompetent DH10MultiBac cells. After electroporation (200 Ω, 25 µF, 2.0 kV pulse), incubate cells for 8 h or overnight at 37 °C and plate on TYE agar containing the selective antibiotics (**Table 2**), IPTG and X-gal, and wrap plates in parafilm.

24| After 20–30 h incubation at 37 °C, white and blue colonies should appear. Pick white clones and proceed to bacmid preparation for insect-cell infection (Step 25).

Preparation of bacmid for insect-cell infection

25| Incubate single clones containing composite MultiBac bacmid overnight at 37 °C in 2 ml of 2× TY liquid medium with the correct combination of antibiotics (**Table 2**).

26| Prepare MultiBac bacmid DNA by alkaline lysis, for example, using solutions I, II and III of the QIAprep Spin Miniprep kit following the protocol provided by the manufacturer. Precipitate the resulting supernatant (~900 µl volume) with 800 µl isopropanol and wash pellet twice with 250 µl of 70% EtOH. Resuspend MultiBac bacmid DNA with 20 µl of filter-sterilized (0.22-µm) ddH_2O.
▲ CRITICAL STEP
■ PAUSE POINT Bacmid DNA in isopropanol can be stored at –20 °C.

Table 2 | Selection marker use

Operation	Vectors	Host strain	Antibiotic[a]	Color marker[b,c]
BD In-Fusion (Step 3)	pFBDM, pFL	TOP10	Amp.	–
	pKDM, pKL	TOP10	Kan.	–
	pUCDM	BW23473	Chl.	–
	pSPL	BW23473	Spec.	–
Double fusion, Cre-mediated (Step 14)	Derivatives of:			
	pFL and pUCDM	TOP10	Amp., chl.	–
	pKL and pUCDM	TOP10	Kan., chl.	–
	pFL and pSPL	TOP10	Amp., spec.	–
	pKL and pSPL	TOP10	Kan., spec.	–
Triple fusion, Cre-mediated (Step 14)	Derivatives of:			
	pFL and pUCDM + pSPL	TOP10	Amp., chl., spec.	–
	pKL and pUCDM + pSPL	TOP10	Kan., chl., spec.	–
Multiplication (Fig. 2c)	Derivatives of:			
	pFBDM, pFL	TOP10	Amp.	–
	pKDM, pKL	TOP10	Kan.	–
	pUCDM	BW23473	Chl.	–
	pSPL	BW23473	Spec.	–
loxP integration (Step 21)	MultiBac bacmid and derivatives of pUCDM	DH10MultiBac^{Cre}	Amp. Kan. Tet. Chl.	X-gal and IPTG
	MultiBac bacmid and derivatives of pSPL	DH10MultiBac^{Cre}	Amp. Kan. Tet. Spec.	X-gal and IPTG
Tn7 transposition (Step 23)	MultiBac bacmid[d], and pFBDM or pFL derivatives[e]	DH10MultiBac	Amp., gent., tet.	X-gal and IPTG
	MultiBac bacmid[d] and pKDM or pKL derivatives[e]	DH10MultiBac	Kan., gent., tet.	X-gal and IPTG

[a]Concentrations: ampicillin (amp.) 100 µg/ml; kanamycin (kan.) 50 µg/ml; chloramphenicol (chl.) 30 µg/ml; spectinomycin (spec.) 50 µg/ml; tetracycline (tet.) 10 µg/ml, gentamycin (gent.) 10 µg/ml. [b]Only on agar plate: IPTG, 0.5 mM; X-gal, 500 µg/ml. [c]–, none. [d]With or without donor derivatives integrated in *loxP* site of bacmid. [e]Including double or triple donor-acceptor fusions.

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Virus generation

27| For every MultiBac bacmid DNA probe, seed 0.5×10^6 freshly diluted *S. frugiperda* Sf21 cells in two wells each of a 6-well tissue culture plate and incubate for 15 min at 27 °C (for cell maintenance see **Box 1**).
Steps 27–34 should be performed in a sterile hood.

28| Combine in Eppendorf tubes 10 µl MultiBac bacmid DNA solution with 5 µl CellFECTIN transfection reagent in 200 µl serum-free medium (Sf-900 II SFM). Incubate for 15 min at 27 °C.
▲ **CRITICAL STEP**

29| Add 1 ml of medium to the CellFECTIN-DNA suspension and use it to replace supernatant from seeded cells. Seal 6-well plate with parafilm and incubate for 5 h at 27 °C. Then, aspirate off suspension and add 3 ml of fresh medium, and again seal with parafilm. Incubate at 27 °C.

30| After 48–60 h (at the latest), collect supernatant and store in a 15-ml Falcon tube. This is the initial virus V_0 (~6 ml).
Optional: 3 ml fresh medium can be added per well if further progression of infection and/or protein production is to be monitored.
▲ **CRITICAL STEP**

Virus amplification and protein production

31| In a 500-ml shaker flask, add 3 ml of V_0 to 50 ml of freshly diluted cells at a density of 0.5×10^6 (cells/ml). Culture cells at 27 °C, with shaking at 80 r.p.m. Monitor cell count every 24 h. Split cells at 24 h intervals to below 1×10^6 until cell proliferation arrests. Simultaneously, swelling of the cells (about twofold) should be observed. After an additional 48 h, collect the supernatant. This is generation 1 virus (V_1). *(Pelleted cells can be resuspended in fresh medium to monitor protein expression;* ***Box 2****.)*
➨ **TROUBLESHOOTING**

BOX 1 MAINTENANCE OF INSECT CELLS

Stock suspension cell culture. Maintain a liquid culture of Sf21 cells in 50 ml of SF-900 II SFM serum-free medium at a cell count of $0.5–1 \times 10^6$ cells/ml in a 500-ml shaker flask at 27 °C, shaking at 80 r.p.m. Check cell count every 24 h using a light microscope. Flasks must be dedicated to insect-cell applications only, and cleaned and stored following standard cell culture–grade protocols.

Growing cells. For virus amplification, cells are seeded from the starter culture in a 2-l shaker flask in 100 ml of medium at a cell count of ~0.5×10^6 cells/ml. The volume should double to 200 ml (after 24 h) and to 400 ml (after 48 h). For protein expression, (Steps 31–33) cells are seeded from one 2-l shaker flask into a desired number of flasks. Fresh medium is supplemented in 24-h steps until a volume of 400 ml is reached per flask. For optimal aeration, cell count should not exceed 1.5×10^6 cells/ml throughout the procedure. If cells continue to double during protein production, it is advisable to split the cultures upon reaching a cell count >1.5×10^6 cells/ml by placing half of the volume into a fresh shaker flask and replenishing an equal amount of fresh medium.

Freezing and thawing cell stocks. A <2-week-old suspension cell culture maintained at $0.5–1 \times 10^6$ cells/ml is centrifuged at 500*g* for 5 min. The cell pellet is gently resuspended in fresh medium to a cell count of 3×10^7 cells/ml and DMSO is added to 10%. Aliquots (3 ml) in 4.5-ml cryo tubes are flash frozen in liquid nitrogen and can be stored for up to several years.

For thawing, a 3-ml aliquot is incubated in a 37 °C water bath and immediately removed when thawed. The cryo tube is then centrifuged at 500*g* for 5 min. Medium is removed, and the cell pellet is gently resuspended in 2 ml of medium and transferred to a 50-ml Erlenmeyer flask. Fresh medium is added to achieve a cell count of 1×10^7 cells/ml. After approximately 4–6 d shaking at 80 r.p.m., cell division should begin and occur every 18 h. After 2 months, cells should divide every 24 h. Fresh stock suspension cultures should be started every 2 months.

Cell line used. We use Sf21 insect cells and Sf-900 II SFM for all steps involving cell culture. It has been reported in the literature that certain cell lines are particularly suited for virus amplification, whereas others are ideal for protein expression. Protein production and virus generation could be optimized by testing various cell lines (Sf9, Hi-5), optionally supplemented with serum. But maintenance of one cell line (Sf21) only for all purposes in serum-free medium reduces handling considerably and in our hands leads to satisfactory results both for virus amplification, storage and protein production in all cases tested. For longer term storage of virus (>100 d), it can be advantageous to add fetal calf serum to 10% to reduce virus aggregation and concomitant titer loss, or to freeze virus aliquots at –80 °C.

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BOX 2 MONITORING PROTEIN EXPRESSION

Expression analysis by SDS-PAGE. Protein expression can be monitored by SDS-PAGE at every step from initial infection to protein expression by production virus. Protein production is often not manifest in cells used for initial transfection on 6-well plates (unless fluorescent markers are used, see below). It is recommended to monitor protein expression starting from cell proliferation arrest in cultures (t = 0 h). Every 12 h (or 24 h), 1 ml sample is withdrawn from the liquid culture. Cells are pelleted for 1 min in an Eppendorf microcentrifuge at 5,000 r.p.m. at 4 °C. Pellets are resuspended directly in 500 µl of SDS protein gel loading buffer with dye or, alternatively, in 250 µl of a lysis buffer of choice (for example, PBS) supplemented by an equal volume of SDS protein gel loading buffer. Short bursts (2–3 s) from a sonicator at low settings reduce viscosity of the sample. SDS-containing samples are boiled for 10 min at 95 °C, centrifuged briefly at 14,000 r.p.m. in an Eppendorf microcentrifuge and cooled on ice for 1 min before loading 5–10 µl on the gel.

Expression analysis using fluorescent markers. We have found it particularly useful to monitor heterologous expression by following the specific signals of fluorescent proteins (for example, eYFP and/or eCFP) cointegrated into the virus with the genes encoding for the proteins of choice. We have observed previously that coexpression of fluorescent proteins did not reduce heterologous protein complex yield considerably, even if large protein complexes of several hundred kilodaltons were expressed[1]. In the virus shown in **Figure 4**, we had cointegrated eCFP in the Tn7 site and eYFP in the *loxP* site of the MultiBac baculoviral genome. From infected liquid culture, 1-ml sample (~1×10^6 cells) was withdrawn and centrifuged at 5,000 r.p.m. in an Eppendorf microcentrifuge for 1 min at 4 °C. Cell pellet was resuspended in 0.5 ml of PBS and treated with brief bursts (2–3 s) from a sonicator at medium levels, followed by centrifugation at 14,000 r.p.m. in an Eppendorf microcentrifuge for 10 min. Expression of fluorescent proteins from both sites was conveniently monitored by fluorescence spectroscopy using the specific fluorescent signatures of eCFP and eYFP[10]. When both signals reached a plateau, the cultures were collected and analyzed by protein SDS-PAGE. High-level fluorescent protein production was concomitant with maximal production of the respective protein complex.

32| In a 2-l shaker flask, add 5–10 ml V_1 in 400 ml cell culture at 0.5×10^6 (cells/ml). Monitor cell count every 24 h. Split cells to maintain a cell count below 1×10^6 until cell proliferation arrests. After an additional 48 h, collect the supernatant. This is generation 2 virus (V_2).
Both V_1 and V_2 can be used for protein production. It is advisable to keep aliquots of V_0 and V_1 stored at 4 °C for regeneration of production virus V_2, if needed.
➡ **TROUBLESHOOTING**
■ **PAUSE POINT** All viruses (V_0, V_1 and V_2) can be stored for several months at 4 °C protected from light. V_0 and V_1 are stored in sterile Falcon tubes. V_2 production virus can be stored conveniently in emptied sterile SF900 II SFM medium bottles.

33| For large-scale expression extending beyond 400 ml cell culture, multiply the setup outlined in Step 32 with further 2-l shaker flasks containing 400 ml of cell culture each. Use production virus V_2 for infection.
*It is advisable to monitor cell division in a pilot experiment with one shaker flask infected with 10–15 ml production virus V_2, as splitting cells below 1×10^6 cells/ml if not enough virus was added is not convenient in this large scale setup. The multiplicity of infection (MOI = ratio of virus particles per cultured cell) should be greater than 1. If cell proliferation continues in the pilot experiment upon virus addition beyond a cell count of 1.5×10^6 (cells/ml), the volume of V_2 virus added in a second pilot experiment should be increased to aim for a MOI > 1. Protein expression is monitored throughout large scale protein production in at least one shaker as described (**Box 2**).*

TROUBLESHOOTING TABLE

PROBLEM	SOLUTION
Step 6 There is unspecific or no PCR product.	The annealing temperature may have to be optimized. Alternatively, annealing temperature can be successively decreased in the first five cycles down from 60 °C to 50 °C by 2 °C/step.
Steps 7, 12 There is high background of parental plasmids in spite of *Dpn*I digestion after transformation.	Purify PCR reaction with QIAprep PCR Purification kit or, alternatively, precipitate PCR reaction with NaOAc-EtOH after phenol-chloroform extraction. Resuspend DNA pellet in ddH_2O supplemented with 10× NEB buffer 4. Digest 10–12 h or overnight with 20–40 U *Dpn*I enzyme. A restriction enzyme which cleaves the parental plasmid but not the PCR product can be added to this reaction.

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Step 16 No transformants were obtained.	Repeat Step 15 with higher concentrations of donor(s) and acceptor plasmid DNA. In particular for triple donor-donor-acceptor fusions, efficiency of fusion is low and more colonies can be obtained by transforming into electrocompetent cells (~10^9 cfu/μg).
Step 20 Cre is not expressed.	Make sure that low-salt agar and medium are used for plating and preparing competent cells (see **Supplementary Methods**). Use freshly prepared L-arabinose solution for induction at OD_{600} = 0.25 (not higher).
Step 22 No transformants were obtained.	Verify Cre expression level in DH10MultiBac^{Cre} cells by SDS-PAGE. If Cre is not highly expressed, prepare new batch of competent cells.
There are several white colonies among blue colonies.	Competent cell batch may be contaminated, since all colonies obtained must be blue at this step. Streak out DH10MultiBac^{Cre} competent cells on 2× TYE plates containing X-gal, IPTG and appropriate antibiotics (**Table 2**) except for resistance marker selecting for donor plasmid. All colonies obtained must be blue.
All colonies are white.	X-gal and/or IPTG in 2× TYE plates may be aged. Replate on agar containing fresh X-gal and IPTG.
Steps 31,32 Cells divide without proliferation arrest.	Initial V_0 (Step 31) or V_1 (Step 32) virus may have low titer. Repeat infections with more virus added. Continue splitting cultures to below 1×10^6 each day for a total of 5–7 d and monitor cell count. If cell culture still divides after 7 d, repeat the initial transfection.
Cell cultures stop dividing immediately upon virus addition.	V_0 or V_1 was added at MOI > 1. This could lead to accumulation of defective virus and loss of protein expression. Repeat infections with less volume of respective virus.
No protein production is observed.	Cells may be aged. Repeat infections with cultures using freshly prepared cells (**Box 1**). Virus may have been overamplified. Start again at Step 31 or 27 using less virus for infection. Safeguard that cells divide once or several times upon virus addition (MOI << 1). Also for amplification take care to collect virus 48 h after infection. Make sure cell culture volumes do not exceed those indicated and thereby restrict aeration.

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CRITICAL STEPS

Step 2 The multiplication module can be used to create multigene expression cassettes (**Fig. 2c**) and also to conveniently generate dual expression cassettes from single gene progenitor plasmids. To be used for multiplication, the restriction sites (*Pme*I, *Spe*I, *Avr*II and/or *Bsp*68I) have to be removed from the gene ensemble, if present. Alternatively, for a complex containing three genes, for example, it may be best to use two single-gene donors and one acceptor derivative joined by *in vitro* Cre fusion.

Step 7 This step is necessary to remove methylated parental DNA and hemimethylated hybrids of one parental and one PCR synthesized strand in the PCR reactions that would otherwise give rise to high background after transformation. Alternatively, the linearization of the vector by PCR can be designed such that one or several restriction enzyme sites that do not cleave the final construct are removed from the expression cassette by placing the homology regions in the respective primers accordingly. Then, background can be suppressed by restriction digestion with the enzyme of choice and/or *Dpn*I.

Step 11 The correct cell type has to be chosen for transforming BD In-Fusion reaction products. Donor plasmids and derivatives contain a conditional origin of replication derived from R6Kγ and have to be propagated in cell strains expressing the *pir* gene such as BW23473 (**Table 1**). Acceptor plasmids and derivatives can be propagated in common laboratory bacterial strains (for example, TOP10 or DH5α).

Step 12 Phusion polymerase has the lowest currently reported error rate. Nonetheless, it is advisable to verify the inserted gene as well as promoter and terminator regions of the constructs by sequencing. As several copies of the same promoters and terminators are often used in a multiprotein expression experiment, it is necessary to sequence individual promoter and terminator regions by 'inside-out' gene-specific primers annealing close to (~100 bp) the 5′ and 3′ end of the genes.

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Step 16 Owing to the conditional origin of replication, donor derivatives are not propagated in *pir*⁻ strains (TOP10, DH5α and others). Cre-fusion reactions of donor and acceptor plasmids that are transformed into strains lacking *pir* give rise to double or triple fusions (**Supplementary Fig. 2**) isolated based on resistance marker combinations (**Table 2**). We evaluated pairwise fusion efficiency by plating on agar plates containing only one antibiotic selecting for the acceptor plasmid, or two antibiotics including the one provided by the donor, thus selecting for fusions. We obtained 12–15% efficiency, which is consistent with previous reports. To further facilitate multigene vector assembly, one can incubate both donors simultaneously with the acceptor in a single reaction (**Fig. 2**). Such fusions are isolated by plating on triple-resistant agar plates (**Table 2**). Although much less efficient than the pairwise reactions, we typically obtained 20–50 colonies from each transformation reaction using 1 μg total DNA and our own $CaCl_2$ competent cells (10^7 c.f.u./μg).

Step 17 Occasionally, *in vitro* Cre-fusion can result in two identical donor derivatives fused to one acceptor (see **Supplementary Fig. 2**). Integration of such fusions into the MultiBac bacmid results in increased protein production levels from the duplicated insert.

Step 18 It is best to occupy the *loxP* site present on the MultiBac plasmid first if integrations both into the Tn7 and *loxP* sites are planned. From positive DH10MultiBac^{Cre} clones, electrocompetent cells containing the recombinant bacmid are then prepared using the resistance markers indicated (**Table 2**) following standard protocols. Those competent cells are used for subsequent transformation with acceptor plasmid derivatives which can contain (optionally) donor derivatives fused by *in vitro* Cre reaction (**Fig. 1**). In principle, it is also possible to charge the Tn7 site in DH10MultiBac cells first (Step 23). In this case, however, plasmid derivatives must be used that do not contain a *loxP* site (pFBDM or pKDM rather then pFL or pKL, see **Supplementary Fig. 1**). Then, positive clones that contain recombinant MultiBac bacmid with genes inserted into the Tn7 site can be used to generate competent cells expressing Cre recombinase (Steps 19, 20). These competent cells are next transformed with donor derivatives.

Step 19 Antibiotic resistance by zeomycin is inhibited by high salt concentrations. Therefore, in both TYE agar plates and in the culture used for preparing Cre-competent cells salt concentrations need to be adjusted accordingly (see Materials and **Supplementary Methods**).

Step 20 For successful integration of donor derivatives into the *loxP* site of the MultiBac bacmid cells, a strong expression of recombinant Cre recombinase must be observed by 15% SDS-PAGE analysis upon induction with arabinose (**Supplementary Methods**) and therefore should be monitored for each batch of competent DH10MultiBac^{Cre} cells produced. Competent cells can be kept at −70 °C for months without a loss of integration efficiency. Batches can be quality controlled by fusing a control plasmid containing a fluorescent protein (for example, pUCDM-eYFP) and monitoring expression by fluorescence spectroscopy already at the level of initial transfection (**Box 2**).

Step 22 The blue color of the colonies indicates that the Tn7 transposition element, which is embedded in a *lacZ* gene, is intact and can be used for integration (**Fig. 1**). All colonies on the plate should develop blue color (see Troubleshooting for Step 22).

Step 26 It is very important to completely remove granular material that forms after cell lysis; otherwise chromosomal DNA and other nucleic acids will contaminate the composite MultiBac bacmid. This can hinder initial transfection (Step 29) since the ratio of total nucleic acid to lipofectant is crucial for success. We recommend adding a second centrifugation step to the QIAprep protocol in fresh Eppendorf tubes. The resulting supernatant should be clear of floating debris, and can be transferred to fresh Eppendorf tubes for isopropanol precipitation.

Step 28 In addition to CellFECTIN, there are several other lipofectants which can be used as well for initial transformation (for example, FuGENE). To date, we have carried out the majority of experiments in this laboratory using CellFECTIN. Transfection with FuGENE in our hands works equally satisfactory and further offers the advantage that the lipofectant suspension does not have to be removed after a 5-h incubation.

Step 30 (also 31,32,33) To maintain MOIs below 1 (ideally closer to 0.1) and thus to avoid drop of protein expression levels through virus generations, it is mandatory to strictly follow the time course presented in Steps 30–33. Initial virus has to be collected after 48–60 h, even if cells do not show clear signs of infection and continue proliferating on the 6-well plate until confluency is reached (cells cover entire well). When using shaker flasks, it is critical not to use cell culture volumes which are too large (500-ml flask, maximum 50 ml;

2-l flask, maximum 400 ml). Also, it is particulary important to carefully control cell density and infect at each corresponding step at a cell count below 1×10^6 (cells/ml) (best between 0.5×10^6 and 0.7×10^6 (cells/ml)). Cell cultures during virus amplification have to be counted and split every 24 h to below 1×10^6 (cells/ml) until cell proliferation stops. Failure to strictly follow these guidelines may result in loss of protein expression due to lack of aeration if volumes and/or cell counts are too high. Upon infection, cells must still be proliferating during the first 24 h, otherwise the number of viruses per cell is above 1 (see Troubleshooting). Then, defective virus accumulates which can result in complete loss of heterologous protein expression.

COMMENTS

Multiprotein complexes with many subunits have become an intensive focus of current biological research. Consequently, several multigene expression systems have been introduced for heterologous complex production. Systems that have been used with success in *E. coli* include the pST44 polycistronic expression system[3] and the Duet system from Novagen[4]. For large eukaryotic complexes, we have recently introduced the MultiBac baculovirus system[1]. In contrast to expression in *E. coli*, proteins which are up to several hundred kilodaltons in size can be produced in insect cells infected with recombinant baculovirus, and the heterologous gene products generally are authentically processed and targeted to their native cellular component. Insect-cell expression using baculoviral vectors is thus a convenient choice for production of large multiprotein complexes, which are the rule rather than the exception in eukaryotic cells[5,6].

The vectors and protocols provided here were designed such that both the initial integration of multiple genes into the baculoviral genome and the subsequent alteration of such gene combinations be as simple and rapid as possible. The modular use of the transfer vectors presented here, and the use of recombination-mediated fusion allows for multigene expression vectors to be reconstructed with ease from progenitor donor and acceptor plasmids containing one or two genes each.

A paramount issue for successful protein production using the baculoviral system is virus stability. In cell culture, it is often observed that deletion genotypes accumulate and eventually dominate the virus population, ultimately resulting in virtually complete loss of heterologous expression[7,8]. Defective virus is preferentially produced in culture when infecting at high ratio of virus to cells or when collecting virus at times when the selective pressures from heterologous protein expression come to bear. Multigene baculoviral vectors based on the MultiBac bacmid can contain numerous repetitive elements (promoters, terminators, recombination sequences) which may represent preferred sites for eliminating recombinant insert *in vivo*. We have analyzed amplified MultiBac viruses both by restriction mapping of purified viral DNA (**Fig. 3**) and at single-cell resolution by immunofluorescence spectroscopy (**Fig. 4**), illustrating the dangers of incorrect amplification of virus. Adherence to the protocol, in contrast, allows for amplification of virus with constant protein production properties.

In principle, virus titer could be verified at every step for instance by classical methods such as plague assay or end-point dilution[9]. The general protocols we provide here are not optimized in this respect, and it is clear that careful determination of individual virus titers and multiplicities of infection may be helpful for protein yield optimization. Our strategy relies on controlling MOI by dilution and splitting of cell cultures, thus avoiding lengthy analytical steps in the process from initial virus to protein production. Our setup is robust and standardized, and thus compatible with approaches in which numerous expression experiments are to be carried out in parallel in a time-saving way. There are many fermentors (for example, Wave Bioreactor, WAVE Biotech LLC; Biostat B, B.Braun Biotech int.) that can be used for large-scale cultivation of insect cells. In our hands, however, the use of ordinary laboratory shaker flasks as described in this protocol provided sufficient yield (~ 1–20 mg/l depending on protein complex) in most cases, even for structural biology applications.

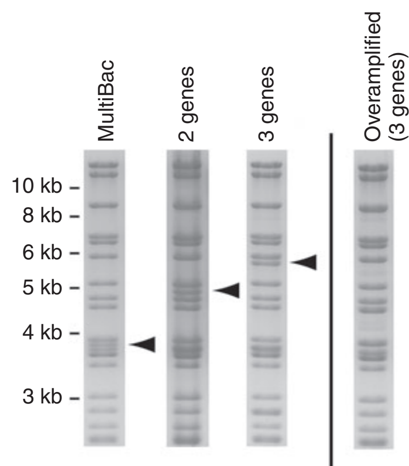

Figure 3 | Stability of MultiBac baculoviral DNA analyzed by ethidium bromide–stained 0.8% agarose gels. *Sal*I-digested MultiBac viral DNA from production virus (V_2) gives the band pattern shown without recombinant insert (lane 1), and with two and three genes, respectively, integrated into the Tn7 site (lanes 2 and 3). Position of bands corresponding to the liberated DNA fragment containing the recombinant insert are marked with arrows (*Sal*I does not cleave in the recombinant insert region). Virus shown in lane 3 was in a separate experiment overamplified by serial passaging at high MOI leading to accumulation of viral DNA lacking the recombinant insert (lane 4).

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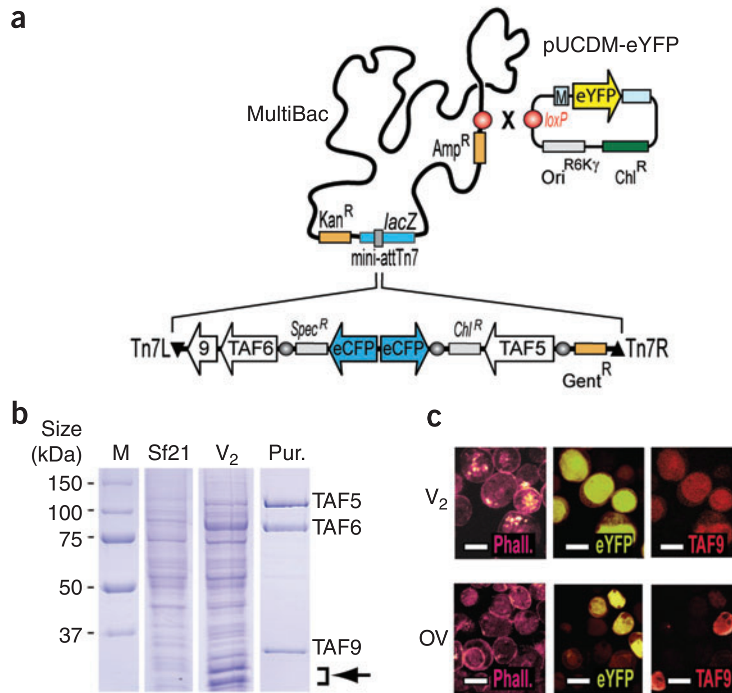

Figure 4 | Protein complex expression by using MultiBac baculoviral vector. (**a**) Six genes were integrated into a MultiBac baculovirus encoding a subcomplex of human TFIID (TAF5, 6 and 9) and fluorescent proteins eCFP (2 copies) and eYFP. (**b**) Protein complex was purified from cell lysates infected with production virus V_2 (SDS-PAGE). The arrow denotes bands corresponding to fluorescent proteins. Virus V_2 was generated as outlined. M, marker. (**c**) Infected cell cultures were assayed for expression of YFP and TAF9 by immunofluorescence. Phalloidin (Phall.) stains both infected and uninfected cells. Cell cultures infected with production virus (V_2) produced the proteins in each cell assayed. Infection of cells with virus serial passaged at MOI >> 1 revealed cells expressing either eYFP or TAF9 indicating that deletion virus had accumulated (OV, overamplified virus). Scale bars, 20 μm.

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Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT
The authors declare competing financial interests (see the *Nature Methods* website for details).

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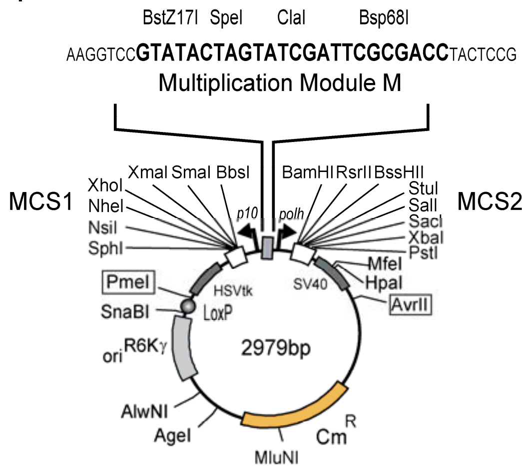
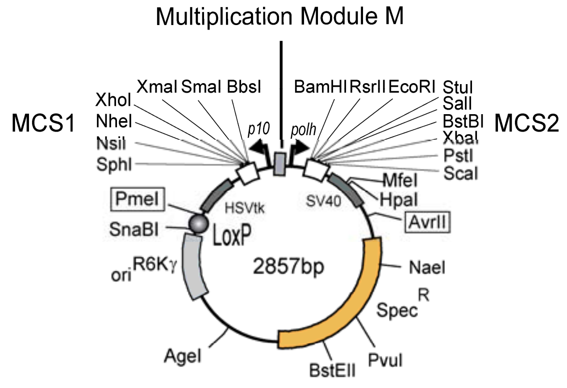
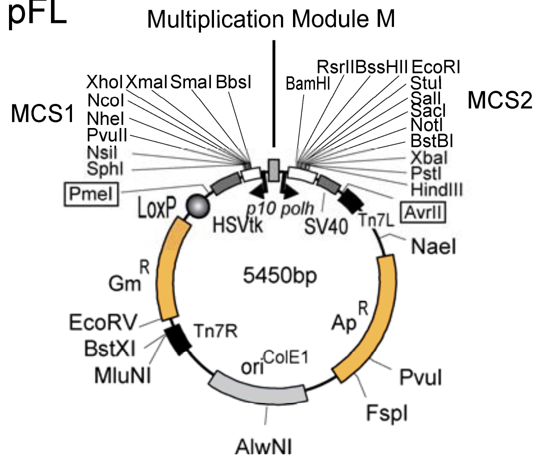
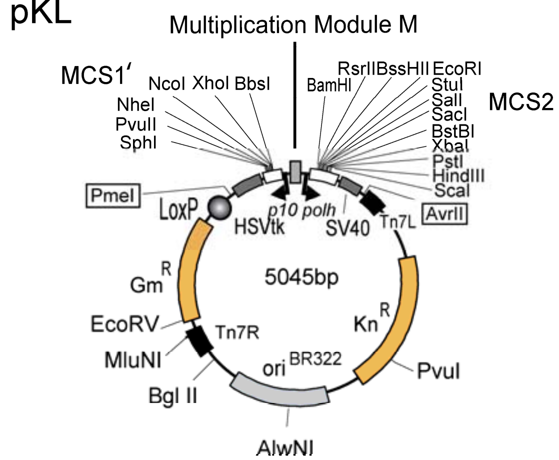
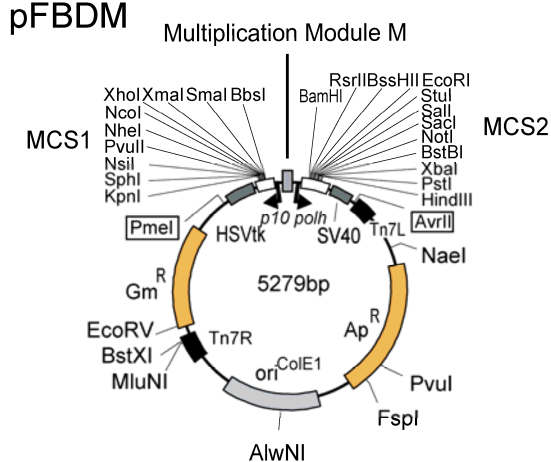
Supplementary Figure 1:

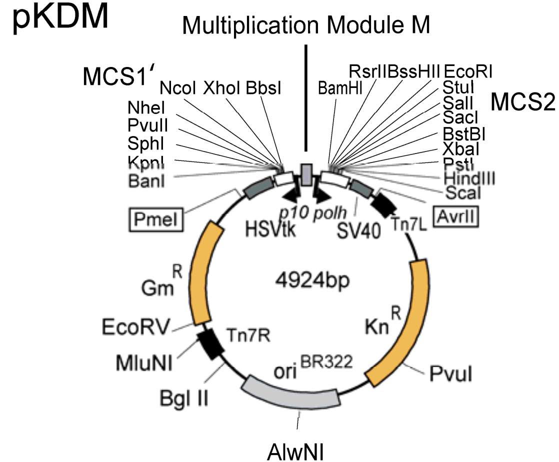








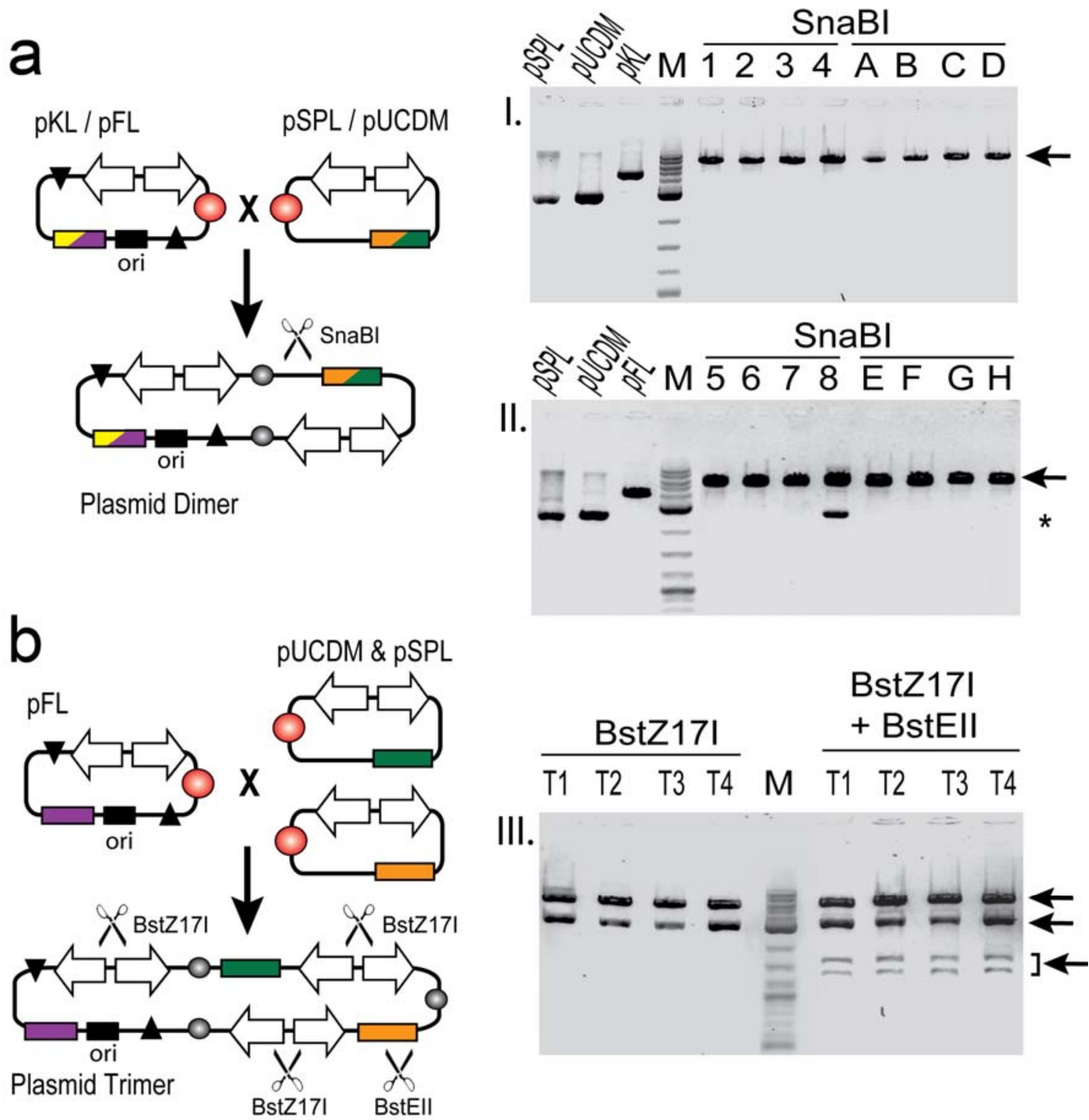




Plasmid maps of MultiBac transfer vectors. Multiple cloning sites (MCS), promoters (polh, p10) and terminators (SV40, HSVtk) are shown. pUCDM and pSPL contain a conditional origin of replication (R6Kγ). pFBDM and pFL contain a high copy-number

replication origin (ColE1). pKDM and pKL have low-copy replication origins derived from pBR322. pFL, pFBDM, pKL and pKDM contain transposon elements (Tn7R, Tn7L), vectors pUCDM, pSPL, pFL and pKL have a LoxP imperfect inverted repeat flanking the dual expression cassette. All vectors contain the previously described multiplication module (M) for generating multigene cassettes[1]. pFL and pKL (and derivatives) are acceptor vectors, pUCDM and pSPL (and derivatives) are donor vectors in Cre mediated plasmid fusions (main text, Fig. 2). Maps were generated with DNAMAN.

Supplementary Figure 2:



Plasmid fusions (**a**) Cre-fusions of donor and acceptor plasmids *in vitro* via LoxP. Four transformants each from reactions with the plasmid combinations indicated (left) were analyzed by SnaBI restriction showing correct fusion reactions in all cases tested (Clones 1-4: pSPL/pKL; A-D: pUCDM/pKL; 5-6: pSPL/pFL; E-H: pUCDM/pFL). The SnaBI site present in the fusions is marked. Corresponding agarose gels are depicted on the right (I, II). Linearized fusions are marked with an arrow. As marker, 1kb DNA

Ladder (NEB) was used. Clone 8 showed a second copy of the donor integrated (asterisk).

(**b**) Transformants from a simultaneous Cre-fusion of both donors to acceptor pFL (left) were analyzed by restriction with BstZ17I alone and in combination with BstEII (respective sites are marked). All clones tested (T1-T4) contained correct fusions with restriction fragments (III, arrows) according to the sizes expected.

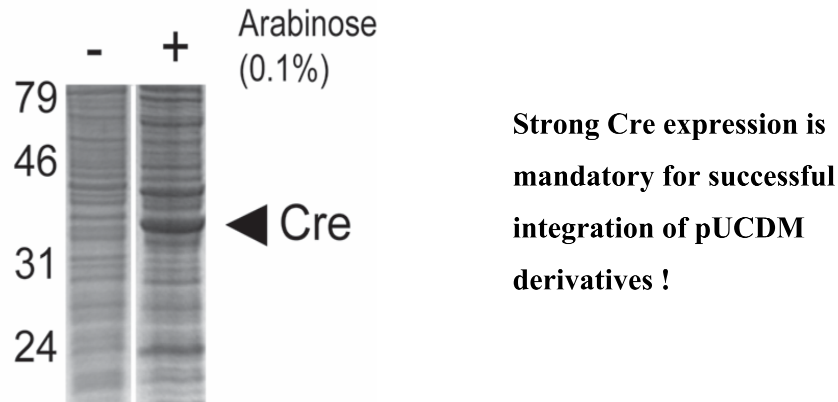
Supplementary Methods:

Generating DH10MultiBac^{Cre} cells.

Expression of Cre Recombinase Protein und generation of electro-competent DH10MultiBac^{Cre} cells:

1. Electroporate pBADZ-HisCre plasmid into DH10MultiBac cells (25 microFD, 2.0 kV, 200 Ohm).
2. Grow in 2xTY medium for 4h at 37°C.
3. Plate on LS plates (low salt medium agar) with antibiotics (kanamycin, tetracyclin, zeocin) and X-Gal/IPTG.
4. Grow a 500 ml LS (low salt) culture from one blue colony. Antibiotics: Kanamycin/tetracyclin/zeocin, Temp. 37°C or RT.
5. Grow to OD_{600}=0.25 at 37°C or RT.
6. Take 500 µl ample („Minus"-probe)
7. Add L-arabinose to 0.1% (0.5 g in 500 ml).
8. Grow to OD_{600}=0.5.
9. Take 250 µl sample („ Plus"-probe)
10. Cool culture on ice for 15 min.
11. Resuspend in 500 ml ICE COLD STERILE 10% glycerol sol.
12. Centrifuge at 4000rpm, 4°C, 15 min.
13. Resuspend in 250 ml ICE COLD STERILE 10% glycerol sol.
14. Centrifuge at 4000rpm, 4°C, 15 min.
15. Resuspend in 10 ml ICE COLD STERILE 10% glycerol sol.
16. Centrifuge at 4000rpm, 4°C, 15 min.
17. Resuspend in 1 ml ICE COLD STERILE 10% glycerol sol.
18. Prepare 80 µl aliquots (sterile Eppendorfs).
19. Shock-freeze in liq. nitrogen, store at –70°C.
20. Centrifuge „Minus"-probe und „Plus" probe (14 krpm, 5 min, RT).
21. Resuspend in 150 ul protein gel loading buffer. Analyze by 15% SDS-PAGE (load 5-10 ul) (Fig. S3)

Cre expression in DH10MultiBac^{Cre} competent cells
(reprinted with permission from Ref. 1)



Strong Cre expression is mandatory for successful integration of pUCDM derivatives !

Low salt medium/Agar for zeocin cultures:

1. Combine 10 g tryptone, 5g NaCl, 5g yeast extract
2. Add water (dd) to 950 ml
3. Adjust pH to 7.5 with 1N NaOH
4. Add water (dd) to 1L (for plates add 15g/L agar) and autoclave.
5. Add zeocin to 25 µg/ml below 55°C (same for other antibiotics)
   X-Gal to 500µg/ml from 1000x stock
   IPTG to 0.5 µM from 1000x stock

Store plates at 4°C in the dark (X-Gal is light sensitive).

Purifying composite MultiBac virus and restriction mapping.

(adapted from Ref. 9):

1. Centrifuge 25ml viral supernatant for 75min in a SW28 rotor at 24.000 rpm through 3mls of a 25% sucrose cushion.
2. Resuspend pellet in 1ml of 10mM Tris (pH 7.5), 100mM NaCl, 10mM EDTA, 0.25%SDS.
3. Incubate at 50ºC for 4h.
4. Extract with phenol/chloroform, NaOAc/EtOH precipitate.
5. Digest with SalI according to manufacturers (NEB) instructions.