

The MultiBac BEVS for producing proteins and their complexes (Prot54)



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Introduction

Protein complexes are central to cellular function. Many important complexes in eukaryotes require recombinant overproduction for a detailed analysis of their structure and function. Baculovirus expression vector systems (BEVS) have become increasingly popular for the production of such specimens, in particular for complexes which depend on a eukaryotic host cell machinery for proper folding, post-translational modification, authentic processing and correct targeting to cell compartments for their activity. The MultiBac system (Fig. 1) is a BEVS specifically designed for producing large eukaryotic complexes with many subunits [Berger et al., *Nat. Biotechnol.* 2004, Fitzgerald et al., *Nat Methods*, 2006, Bieniossek et al., *Trends Biochem. Sci.* 2012]. It consists of an array of plasmids which facilitates multigene assembly, a modified baculovirus genome with optimized protein production properties, and a set of protocols detailing every step from inserting encoding DNAs in the plasmid array to protein production by this technology. The components of the system and the protocols used are continuously being improved, developed and streamlined to simplify handling and improve efficacy [Trowitzsch et al., *J. Struct. Biol.* 2010, Vijayachandran et al., *J. Struct. Biol.* 2011]. We believe that our efforts have reduced the previously perceived complexity of the baculovirus/insect cell system to the level of protein expression in *E. coli*. Detailed protocols are available for utilizing the MultiBac system [Fitzgerald et al., *Nat. Methods* 2006; Bieniossek et al., *Curr. Protoc. Prot. Sci.* 2008], although certain details have been altered over the years and are bound to be refined in the future. In this protocol, we present the procedures as they are currently in operation at the MultiBac platform of Eukaryotic Expression Facility (EEF) at the EMBL Grenoble (Fig. 2), as part of the trans-national access scheme in the P-CUBE (www.p-cube.eu) and BioSTRUCT-X (www.biostruct-x.eu) projects of the European Commission, Framework Program 7. The protocol has been designed such that it can be performed successfully by scientists with or without previous knowledge of or experience with BEVS for expressing their targets during a ten working-day visit at our MultiBac platform.

Parts of the protocols are specific for MultiBac (plasmid fusions, multigene generation, fluorescence tracking). However, all virus amplification and protein production protocols are generically applicable to other baculovirus systems that utilize recombinant bacmids (for example Bac-to-Bac, Invitrogen).

Procedures:

I. Gene insertion into MultiBac plasmids

The MultiBac system has a modular design which facilitates assembling many genes into a single MultiBac baculovirus. This composite multigene virus then produces all genes in every infected cell in an insect cell culture. The logic of assembly involves insertion of the genes into small DNA plasmids called Acceptors and Donors (Fig. 1). The genes can be inserted into the multiple cloning sites of the plasmids by whichever method the user feels most comfortable with. We in the Berger laboratory prefer sequence and ligation independent cloning methods (SLIC) which are particularly useful for large (several kb) DNA insertions. However, conventional methods using restriction enzymes and ligases can be applied as well.

Protocols for either of these options have been described in much detail in our publications [Bieniossek et al., *Curr. Protoc. Prot. Sci.* 2008], also for high-throughput applications [Bieniossek et al., *Nat. Methods* 2009, Nie et al., *Nat. Protocols* 2009] and can be obtained from there.

Important: The plasmids that are part of the MultiBac system, and likewise also the MultiBac virus, have undergone modification and evolution over the years (Fig. 1). Nonetheless, all plasmids and viruses are compatible and can be used together in an expression experiment. Furthermore, all **older baculovirus expression plasmids** that contain the required elements of the Tn7 transposon, Tn7L and Tn7R (notably pFastBac, pFastBacDual and derivatives thereof) **can be used in conjunction with the MultiBac viruses**, for example to improve the quality of the recombinant protein product. If such plasmids are available, there is **NO NEED to reclone** into the MultiBac plasmids, to carry out a production experiment. Instead, these plasmids can be combined with the MultiBac virus genome following protocols provided below.

II. Acceptor-Donor recombineering

The MultiBac system follows a parallel logic for integrating several genes into a single multigene expression plasmid, in contrast to largely serial one-gene-at-a-time approaches which are more common (Fig. 3). This is achieved by fusing one or several Donors, each with one or several genes inserted, to one Acceptor with further genes by a Cre-oxP fusion reaction. The Acceptor plasmid contains the Tn7R and Tn7L elements for integration into the MultiBac virus. The LoxP sites are arranged such that all recombinant DNA cargo will be flanked by the Tn7R and Tn7L sequences in the multigene fusion plasmid (Fig 3).

Important: In the Cre reaction, the Acceptor plasmid must be limiting (i.e. its concentration lower than those of the Donors) to avoid integration of more than one Acceptor into the fusion. Otherwise, more than one Tn7L-Tn7R pair will exist on the fusion plasmid, compromising integration of the recombinant cargo into the MultiBac baculovirus.

We strongly recommend to generate ALL DNA CONSTRUCTS *in silico* prior to carrying out the reactions to avoid later pitfalls.

P1: Cre reaction protocol: ([see comment 1](#)).

Following ingredients are mixed together in an Eppendorf tube

(four plasmid reaction as an example):

10.0 µl ddH₂O
1.0 µl 10xCre reaction buffer
2.0 µl Donor plasmid 1 (~500 ng)
2.0 µl Donor plasmid 2 (~500 ng)
2.0 µl Donor plasmid 3 (~500 ng)
2.0 µl Acceptor plasmid (~400 ng)
1.0 µl Cre recombinase

Incubate at 30°C for 1-2 hours

Streak out on plates with appropriate combinations of antibiotics.

- If only fewer or more Donors are to be fused to one Acceptor, then the volume of ddH₂O must be adjusted accordingly.
- Make sure concentration of each Donor exceeds concentration of Acceptor
- Remember: The Cre reaction is an equilibrium reaction of fusion and excision, so longer incubation will not necessarily increase productive catenation of plasmids.
- The Cre reaction is followed by transformation into regular (i.e. *pir* negative) bacterial cells used for cloning.
- The efficiency of plasmid fusion decreases with growing number of plasmids to be fused. Three-plasmid fusions (for example one acceptor, two donors) work very well in our hands. If four plasmids or more are involved we recommend a sequential approach of a three-plasmid fusion reaction followed by adding further plasmids to purified triple-fusion plasmids by Cre-LoxP reaction.
- We recommend streaking out on plates containing one, two three etc antibiotics (depending on the number of plasmids fused). Then, for example in a four plasmid fusion, successful intermediates (two, three plasmids fused) can be recovered even if the complete fusion reaction of all plasmids fails.

- We recommend testing the clones obtained in a 96-well microtiter plate format to confirm proper resistance marker combinations (detailed in Bieniossek et al., *Nat. Methods*, 2009). Restriction digestion of fusion constructions with appropriate enzymes to confirm presence of all heterologous genes is also highly recommended (using the sequences generated *in silico*).

III. Multigene transfer plasmid integration into the MultiBac baculoviral genome

The chosen multigene fusion plasmid is integrated into the baculovirus genome through transformation into *E.coli* cells containing this genome as a bacterial artificial chromosome. The MultiBac system is Tn7 based, therefore, the *E.coli* cells contain a helper plasmid (Tet resistant) that expresses the Tn7 transposon complex upon induction with IPTG. Selection of positive integrands occurs via blue-white screening – positive clones are white. This is the result of disrupting a LacZ α gene on the baculovirus genome upon productive integration of fusion plasmid.

Table I lists currently available viruses that are compatible with the MultiBac system:

Virus	Cell strain	Specifications	Antibiotics	Comments
Bac-to-Bac (Invitrogen)	DH10Bac	viral genome as BAC helper plasmid for Tn7 transposon enzyme blue-white screening	Kanamycin Tetracyclin	developed in 1980s expresses protease and apoptotic factors
MultiBac	DH10MultiBac	viral genome as BAC helper plasmid for Tn7 transposon enzyme blue-white screening	Kanamycin Tetracyclin	protease deficient chitinase deficient extended cell viability reduced proteolysis
MultiBac ^{Cre}	DH10MultiBac ^{Cre}	viral genome as BAC helper plasmid for Tn7 transposon enzyme helper plasmid for Cre recombinase enzyme blue-white screening	Kanamycin Tetracyclin, Zeocin	protease deficient chitinase deficient extended cell viability reduced proteolysis used to access LoxP site on viral backbone <i>in vivo</i> by Cre
EMBacY	DH10EMBacY	viral genome as BAC helper plasmid for Tn7 transposon enzyme YFP reporter gene in	Kanamycin Tetracyclin Chloramphenicol	protease deficient chitinase deficient extended cell viability reduced proteolysis

		virus backbone blue-white screening		YFP reporter to monitor virus performance and protein production
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Competent cells containing these baculoviral genomes and the helper plasmids are prepared for transformation with MultiBac (fusion) plasmids following standard procedures. We use chemical competent cells.

Integration of transfer plasmids into viruses follows procedure P2.

P2: Integrating MultiBac plasmids into baculoviral genome by Tn7 transposition:

1. (**Day 1**) Following ingredients are added into an Eppendorf tube
 - 5-10 µl plasmid solution containing 10-100 ng MultiBac fusion plasmid
 - 50-100 µl chemical-competent cell solution (DH10Bac, DH10MultiBac, etc...)
2. Mix gently by tapping on table.
3. Incubate on ice for 45 minutes.
4. Heat shock for 45 seconds at 42°C.
5. Add 600 µl of LB (or as preferred).
6. Incubate cell solution at 37°C overnight (8 hours) ([see comment 2](#)).
7. (**Day 2**) Streak out 150 µl on plates containing the antibiotics listed in Table I, Gentamycin, IPTG (1mM) and Bluogal or X-Gal at standard concentration. Use dilution series (1:1, 1:10, 1:100, 1:1000), this results usually in optimal separation of colonies on one of the plates.
8. Incubate plates at 37°C until blue and white colour of colonies can be unambiguously assigned ([see comment 3](#)).
9. (**Day 3**) Pick 2 white clones for each construct, start minicultures overnight (see below) and (optionally) restreak.

We typically pick white colonies from a plate with clear separation of colonies and immediately incubate a miniculture (see P3 below). Simultaneously, that same colony is restreaked on a fresh plate with antibiotics and IPTG/colour reagents to confirm white phenotype while miniculture is growing.

IV. Initial virus generation

Initial virus is generated by purifying composite MultiBac baculovirus from pelleted bacterial minicultures following procedure P3. This preparation is essentially an alkaline lysis of

bacteria. We use solutions from commercially available plasmid preparation kits at this step. These can be for example the Qiagen solutions P1 (buffer with RNase), P2 (alkaline lysis buffer) and N3 (neutralization buffer), however, any other commercial kit can be scavenged for his purpose, or buffers prepared according to any own recipe.

P3: Preparing initial virus, Part 1 (non-sterile work).

1. (**Day 4**) Densely grown minicultures are pelleted by centrifugation. Minicultures are composed of 2 ml media (we use LB) with antibiotics Kanamycin and Gentamycin (Tetracycline can be added is however not really necessary as the presence of the helper plasmid is no longer required). Two white clones each for every construct (see above, P2) are used to inoculate the minicultures. Pellets can be used fresh (preferred), or, alternatively, stored at -20°C for indefinite time.
2. Pellets are resuspended by adding 300 µl P1 buffer, then 300 µl P2 and finally 300 µl N3 buffer (Qiagen nomenclature) according to any generic plasmid preparation protocol for cell lysis.
3. The solution containing white granular material is centrifuged for 10 min at full speed (13-15k) in an Eppendorf centrifuge at RT.
4. Supernatant is transferred to a fresh tube, and centrifuged again at full speed for 3 min. Supernatant is carefully transferred to a fresh tube.

In our experience it is absolutely necessary to ascertain at this step that all debris from the cell lysis is completely removed. Left over debris, containing also genomic nucleic acid and RNA, can inhibit the transfection of insect cells by sequestering transfection reagent.

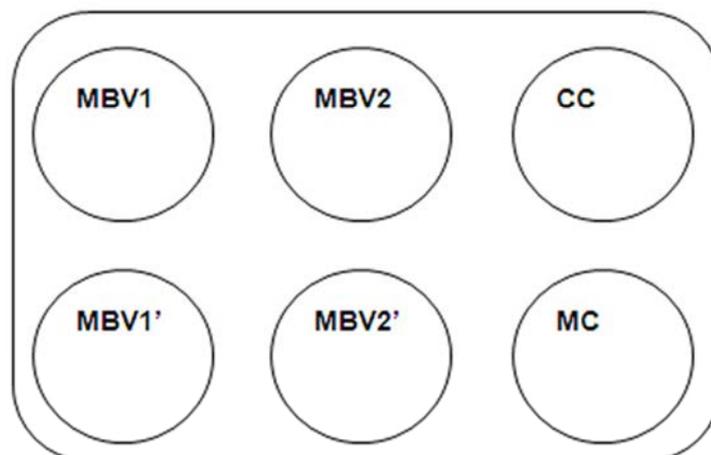
5. Add 700 µl isopropanol to cleared supernatant. Centrifuge at full speed for 10 min.
6. Remove supernatant. Pellet is typically NOT (or hardly) visible. If a solid white pellet is observed, this may indicate incomplete removal of debris (see step 4).
7. Add 200 µl 70% ethanol to wash pellet.
8. Centrifuge at full speed for 5 min (RT or 4°C).
9. Add 50 µl 70% ethanol. Close Eppendorf tupe. Move to STERILE HOOD.

From this step on it is mandatory to work under sterile conditions. The pellet is sterile after precipitation and wash with 70% ethanol. Failure to maintain sterility will result in contamination of the transfected cells.

P3: Preparing initial virus, Part 2 (sterile work).

10. Open Eppendorf tubes in sterile hood and remove ethanol. Dry pellet for 10 min.
11. Add 30 μ l sterile filtered ddH₂O. Dissolve pellet by gently tapping the tube. Allow to resuspend for 10 min. Optional: Transfer 10 μ l of suspension to fresh Eppendorf tube for checking DNA preparation on an agarose gel if desired.
12. Add 200 μ l insect cell media into each tube with resuspended MultiBac baculoviral DNA.
13. Prepare transfection reagent solution by mixing in a separate Eppendorf tube multiples of (for each MultiBac virus to be transfected) 100 μ l media with 10 μ l transfection reagent (we use X-Treme GENE HP transfection reagent from Roche). I.e. for 3 MultiBac viruses 300 μ l media and 30 μ l X-Treme GENE.
14. Add 100 μ l of this mixture to every Eppendorf tube containing dissolved MultiBac baculoviral DNA supplemented with 200 μ l media (step 12).
15. Seed 0.5 to 1 Mio cells from a stock culture into the wells of a 6 well tissue culture (TC) plate. For each MultiBac baculoviral DNA, seed two wells. Supplement media in each well to a total volume of 3 ml.
16. Add half of the MultiBac DNA / X-Treme GENE transfection mixes from step 14 to each of the two wells earmarked for one construct to be tested.

A typical such 6 well plate is shown below in a schematic drawing. MBV1 and MBV1' are wells infected with one MultiBac viral construct (MBV1) MBV2 and BMV2' are wells infected with a different MultiBac viral construct (MBV2). One well contains only cells (CC), and one well only media (MC). These are controls that will help identify the source of any contamination that may occur.



17. Plates are incubated for 60 hours. Then, collect supernatant from the wells, and combine the respective two aliquots (MBV1 and MBV1', for example). This is **V0 virus**.
18. Add 3 ml of fresh media to each well and proceed with plate to procedure **P4**.

We strongly advise to harvest at 60 hours post infection. This is in contrast to most older baculovirus protocols which would allow incubation for 5 days or more. Our aim here is to harvest high-quality budded virus (which may be low titer) which has not yet suffered from damages that can occur through overamplification by allowing for several infection cycles. See also Fitzgerald et al., *Nat. Methods*, 2006, where the quality drop by overamplification was followed experimentally.

P4: Processing dpa+X samples for SDS-PAGE and YFP measurement (if EMBacY is used).

1. To each pellet in Eppendorf tube (dpa, dpa+12, dpa+24, etc) add 500 μ l of PBS (or other buffer of choice, if for example the optimal lysis buffer has been determined already for the particular protein of choice).
2. Sonicate at medium setting for 5-10 seconds until the pellet is completely dissolved (do not overheat!).
3. Remove 50 μ l, transfer into fresh Eppendorf tube, add SDS-PAGE loading dye. This is the "dpa+X SNP" sample (SNP stands for supernatant and pellet, i.e. the whole cell extract, sonicated).
4. Centrifuge remainder of sample (450 μ l) for 3 min at maximum speed in Eppendorf centrifuge (RT).
5. Remove 50 μ l, transfer into fresh Eppendorf tube, add SDS-PAGE loading dye. This is the "dpa+X SN" sample (SN stands for supernatant only, i.e. cleared lysate after sonication).
6. Use remaining 400 μ l to measure YFP fluorescence (excitation: 488 nm, emission max: ~520 nm) if EMBacY baculovirus is used (YFP gene in backbone).

We advise to measure against a standard to calibrate the measurements, and thus render them comparable also with other measurements with other viruses. This allows then quantitative comparisons as long as the samples withdrawn from the expression cultures are always 1 Mio cells (see P5, step 4).

V. Virus amplification & Protein expression test experiment

Virus amplification and concomitant small scale protein test expression is carried out in the same small culture infected with V0 virus following Procedure P4. To save time, this can be started immediately after V0 has been harvested (P3, Step 17).

P5: Amplifying virus & protein expression test.

1. Prepare Erlenmeyer flasks containing 25 ml cell suspension at a density of 0.5 – 1 Mio cells/ml. Make sure cells divide properly in the flasks used (test once or twice the doubling rate, which should be around 18-20 hours for most insect cells at 27°C).

All cell culture experiments are carried out at 27°C, either in incubators or in a room set at this temperature. Insect cells can sustain a certain drop in temperature below 27°C and remain viable (with slower doubling rate). Insect cells can NOT sustain an increase in temperature, they will cease growing (i.e. die) when exposed to temperature increase ([see comment 4](#)).

2. Infect 25 ml cell culture with 3 ml of V0 virus.
3. Monitor cell growth by withdrawing aliquots (12 to 24 hour intervals) and counting cells. If cells double, dilute culture (and split in fresh flask if necessary). Cell density in this culture must remain at 1-1.5 Mio cells/ml to prevent oxygen deprivation at this stage.

Cell cultures infected in this way almost invariably double once (after 18-20 hours) in our hands. This is an indication of the relatively low titer of the virus used. One doubling can be compensated in the shaker flask by topping up to 50 ml. If further doubling is observed (indicative of very weak V0 virus), the culture needs to be diluted and split using a fresh flask or suspension discarded. If cells continue to double after 4-5 days, we recommend to repeat the initial transfection reaction (P3).

4. Determine time when cells stop doubling (day of proliferation arrest, **dpa**). Withdraw 1 Mio cells (i.e. 0.9 ml culture if cell count is at 1.1 Mio/ml). Spin cells at full speed in Eppendorf centrifuge for 1 minute. Discard supernatant and save pellet (-20°C.) this is the “dpa” probe.
Take similar probes (1 Mio cells) every 12 or 24 hours after dpa (dpa+12, dpa+24 etc).

In this way, all downstream experiments to test protein production and/or YFP expression (if EMBacY virus is used) from these samples are calibrated to exactly 1 Mio cells.

5. 48-60 hours after dpa, suspension is transferred to a sterile 50 ml Falcon tube and centrifuged gently (100-150 rcf, 3 min). The supernatant is withdrawn and stored in a fresh sterile 50 ml Falcon tube. This is **V1 virus**. The pellet is gently resuspended in fresh media (50ml) and placed back into the shaker flask.
6. Continue to count cells and to withdraw 1 Mio cells until dpa+96 hours, or, if EMBacY virus is used, until the YFP expression reaches a plateau.
7. Harvest cell pellet (100-150 rcf, 3 min), remove supernatant and save the pellet.

Evidently, pellets will contain intracellularly produced proteins only. Therefore, if secreted proteins are made, pellets AND supernatant need to be stored for further processing.

8. Analyze dpa, dpa+12, dpa+24, etc probes for protein production by SDS-PAGE analysis. For this purpose, dissolve the pellets (1 Mio cells each) in 500 µl PBS (or any other buffer of choice), mix with SDS-PAGE loading buffer, and load on SDS-PAGE gel. If EMBacY virus was used, record also YFP fluorescence of cleared lysates, optionally against a standard YFP probe.

VI. Storing virus as BIICs

Storage of virus is an issue for several reasons. Virus stored as a liquid (in media) has been shown to be prone to deterioration and loss of titer (can be dramatic). Also, flasks occupy space and can fill up 4°C fridges quickly. We prefer by far to store viruses in form of BIICs, i.e. as “baculovirus-infected insect cells”. This method was introduced by D.J. Wasilko and co-workers.

Below are our home-made procedures P6 and P7 derived from D.J. Wasilko’s work which we now standardly use to store viruses. Small aliquots of highly concentrated virus are stored in cryo-vials in liquid nitrogen, occupying much less space than liquid virus. Further, we have not observed deterioration in viral titers for several years when using BIICs.

P6: Preparing frozen baculovirus-infected insect cells (BIIC) stocks,

This procedure is for preparing 5 aliquots of 1 ml BIIC from 50 ml culture. It can be upscaled as required.

1. Grow 50 ml culture of Sf21 cells until 1*Mio cells/ml.
2. Infect cells with a chosen volume of virus.

3. Maintain cells to a concentration of 1 Mio cells/ml until dpa (see P5, step 4). ([see comment 5](#)).
4. Prepare freezing solution containing 90% media (GIBCO SF-900 II SFM, Hyclone, others), 10 g/l BSA, 10% of DMSO. Sterile filter solution.
5. Centrifuge cell culture in sterile 50 ml Falcon tube at 100-150 rcf for 10 minutes, remove supernatant.
6. Resuspend cells gently in sterile freezing solution to a final density of $1 \cdot 10^7$ cells/ml.
7. Transfer 1 ml aliquots into sterile cryovials.
8. Place at -20°C for 1 hour.
9. Store at -80°C for 24 - 48 hours.
10. Store cryovials in liquid nitrogen for indefinite time.

P7: Using BIIC stocks for protein production.

(1 ml of BIIC for 1 l of expression culture)

1. Rapidly thaw one BIIC cryovial in a 37°C water bath (or thaw in your hands, then use paper towels to protect your skin) with gentle agitation until cells are almost thawed.
2. Dilute the vial quickly into 100 ml media (GIBCO SF-900 II SFM, Hyclone, others)
3. Add this 100 ml suspension to 900 ml of uninfected Sf21 cells at a density of around 0.9 Mio cells/ml,
4. Maintain cells at a density of $1 \cdot 10^6$ cells/ml until dpa (see P5, step 4)
5. Harvest cells at proper time after dpa (if EMBacY is used: when YFP reaches a plateau) and purify your protein.

VII. Remarks on protein production, upscaling

The procedure described above for virus amplification & protein expression test (P4) can likewise be used to produce protein, with the difference that the removal of virus at 48 – 60 hours then is no longer performed (P5, step 5). The procedure can be upscaled, then virus V1 (or further expanded virus, V2, V3 if larger volumes are required) is used. In our hands, 50-100 ml of V1 virus often are sufficient for preparing sufficient material even for crystallography, this may certainly vary with the protein species purified.

We very strongly advise to test every virus (V0, V1, V2, etc) for protein production, and also to test BIICs prepared from these viruses by sacrificing one BIIC aliquot immediately when BIIC stocks are prepared. Only if the production properties of a given virus are experimentally tested, can one be sure that there are no unpleasant surprises, for instance if BIICs are used maybe years after they were generated.

During protein production runs, a cell culture may stop proliferation immediately when infected. Then, it may be useful to restart the production experiment and decrease the volume of virus added for infection. Conversely, if the suspension culture does not stop dividing for longer times after infection, then the volume of virus used for infection needs to be augmented. In any case we advise to determine and rely strictly on protein production levels as a definite metric for success in any expression experiment at any scale, rather than optimizing virus volumes and cell culture volumes or cell lines used based on other parameters such as doubling rates, appearance of cells, and others.

Following virus performance and protein production is particularly straight forward if our EMBacY virus is used (Trowitzsch et al., *J. Struct. Biol.* 2010). Then, YFP fluorescence can be used as a means to determine maximum protein production (YFP signal reaches a plateau) and this time of harvest in “real time” by withdrawing probes (1 Mio cells) at certain time intervals (every 12 or 24 hours) from the expression culture.

IIX. Freezing and thawing of cell stocks

We advise to save frozen aliquots of freshly dividing cells, from which then seed stocks can be replenished every so often. The often cited rule of thumb is that a seed stock culture should only be passed for around 60 generations (roughly two months) before a new seed stock needs to be started. We made good experience with keeping seed cultures running for 3-4 months, closely monitoring growth rates. This may not be the upper limit. Nonetheless, it is advisable to start new cell cultures from time to time (Procedure P8).

P8: Freezing and thawing of cell stocks, Part 1 (freezing)

1. Count cells, be sure that you have enough cells for preparing 2-4 vials (see Table II below).
2. Prepare cryovials, cool on ice.
3. Centrifuge cells at 100-150 rcf for 10 min at RT.
4. Remove supernatant.

Remark: If High Five cells are used, make sure to keep the conditioned media when preparing freezing media.

5. Resuspend cells at the density indicated in Table II in the proper media.
6. Transfer 1 ml each to a sterile cryovial.
7. Place at -20°C for 1 hour, then store at -80°C for 24-48 hours.
8. Transfer to dewars filled with liquid nitrogen for long-term storage.

Table II: Media composition for freezing most commonly used cell lines.		
Cell line	Freezing media	Cell density
Sf21, Sf9	60% Sf900 media (GIBCO) 30% Fetal Bovine Serum (FBS) 10% DMSO	1×10^7 cells/ml
High Five	42.5% conditioned Express5 media 42.5% fresh Express5 media 5% FBS 10% DMSO	3×10^6 cells/ml

P8: Freezing and thawing of cell stocks, Part 2 (thawing)

1. Remove vial from liquid nitrogen and place in water bath at 37°C (see comment 6).
2. Thaw rapidly with gentle agitation until cells are almost thawed and remove cells from the water bath
!! Leaving cells at 37°C after they have thawed will result in cell death !!
3. Quickly decontaminate the outside of the vial by spraying with 70% ethanol, dry the vial and place on ice.
4. Pre-wet a 25 cm² TC flask by coating the adherent surface with 4 ml media.
5. Transfer the 1 ml thawed cell suspension directly into this 4 ml of media.
6. Incubate flask at 27°C and allow cells to attach for 30-45 minutes.
7. After cells are attached, gently remove the medium (as soon as possible to remove the DMSO from the freezing solution).
8. Feed cells with 5 ml fresh media.
9. After 24 hours, change to fresh media.
10. Let cells grow until confluence is reached (entire adherent surface of the TC flask covered), then remove cells gently (by shaking) and initiate suspension culture in an Erlenmeyer shaker flask.

P9. DH10MultiBac resp. DH10EMBacY chemical-competent cell generation

1. Streak out DH10MultiBac or DH10EMBacY cells (the latter contain YFP in the bacmid backbone as a marker) from frozen stock on LB + agar plates with antibiotics (Kan, Tet) and BluoGal/ IPTG.
All colonies must be BLUE (can take 24-30 hours). If there are white colonies, the frozen stock is contaminated.
2. Grow *E.coli* strain O/N at 37°C.

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3. Grow few colonies in 20 ml LB at 37°C O/N.
4. Culture 13 ml into 100 ml freshly prepared LB (OD 600 should be around 0.2).
5. Shake suspension at 37°C at 180 rpm for app. 1 h our (depending on cell strain) until OD600=0.5-0.6.
6. Collect the culture in 50 ml centrifuge tubes (or Falcon tubes).
7. Centrifuge at 3000 rcf at 4°C for 5-10 min.
8. Discard the supernatant completely.
9. Add 10 ml of ice cold 0.1M CaCl₂ (keep cold).
10. Resuspend gently on ice.
11. Keep the solution in ice for 15 min.
12. Centrifuge again at 3000 rcf at 4°C for 5-10 min.
13. Completely discard the supernatant and keep for drying.
14. Add 2.8 ml ice cold 0.1M CaCl₂ and mix gently on ice.
15. Add 1.2 ml of sterile ice cold 80% glycerol to each tube with 2.8 ml cell suspension and gently mix.
16. Transfer 100 µl to each sterile Eppendorf tube (use gloves and work next to flame), keep Eppendorf tubes in ice.
17. Freeze tubes in liquid Nitrogen and store at -80°C.
18. Test cells by streakout (!!) from one frozen aliquot on LB + agar plate containing antibiotics (Kan, Tet) and BluoGal/ IPTG.

All colonies must be BLUE (can take 24-30 hours). If there are white colonies, the competent cells are unusable and must be remade.

Materials and Reagents

Plasmids and viruses:

MultiBac reagents (Donors, Acceptors, viruses) and their functionalities are described in detail in our publications, notably in Berger et al., *Nat. Biotechnol.*, 2004; Fitzgerald et al., *Nat. Methods*, 2006 (Supplement contains plasmid maps), Bieniossek et al., *Curr. Protocols Prot. Sci.* 2008; Vijayachandran et al., *J. Struct. Biol.* 2011.

Bacterial Strains:

PirLC, PirHC: These are special cloning strains used to propagate Donor plasmids (such as pIDC, pIDK, pIDS) and their derivatives. They are B-type *E.coli* strains, and contain a *pir* gene that has been inserted into their genome. The *pir* gene is a part of a conditional

replicon derived from R6K γ phage. Donors contain a DNA sequence that requires the *pir* gene product, which is the II protein, for their propagation, otherwise they are not propagated and thus cannot confer antibiotic resistance (suicide vectors). PirLC strains propagate Donor plasmids at low copy number, PirHC strains at high copy number, due to mutations in the *pir* gene. PirHC can be conveniently used to produce empty Donor plasmids in large amounts, PirLC strains are useful for propagating difficult genes cloned into the Donor plasmids.

DH10MultiBac, DH10MultiBac^{Cre}, DH10EMBacY: These are bacterial strains containing the MultiBac baculovirus variants as artificial chromosomes. They are detailed in Table I (above).

Chemicals and reagents:

Bluo-Gal (Invitrogen), Reference: 15519-028 (1g)

Tetracycline (Euromedex) (1000X at 10mg/ml), Reference: UT2965-B (25g)

Kanamycine (Euromedex) (1000X at 50mg/ml), Reference: EU0420 (25g)

Gentamycine (SIGMA) (1000X at 10mg/ml), Reference: G3632 (5g)

IPTG (Euromedex) (1000X at 1M), Reference: EU0008-B (5g)

Cre-recombinase (New England BioLabs), Reference: M0298

Transfection Reagent:

X-Treme GENE HP transfection reagent (Roche), Reference: 06 366 236 001

Insect cells, serum free media:

Hyclone SFM4 Insect (Thermo Scientific), Reference: SH 30913.02

Authors notes:

The MultiBac system since its inception in 2004 has undergone significant reengineering and stream-lining both in protocols and reagents, We had initially designed only two plasmids (pFBDM, pUCDM) which would enter the original MultiBac virus through two sites, a Tn7 attachment site and a distal LoxP site [Berger et al., *Nat. Biotechnol.* 2004]. These original, somewhat crudely crafted reagents have been superseded by more sophisticated plasmids and viruses over the years. We first generated more plasmids (pFL, pKL, pSPL, see Fitzgerald et al, *Nat. Methods* 2006) to facilitate multigene construction. More recently, we added newly synthesized, smaller plasmids to this growing array (pACEBac1, pACEBac2, pIDK, pIDS, pIDC, Vijayachandran et al., *J. Struct. Biol.* 2011) which are compatible with

automation using tandem recombineering procedures we developed (Bieniossek et al., *Nat. Methods* 2009, Nie et al., *Nat. Protocols* 2009). Importantly, all plasmids are compatible with each other, and also with integration into the various baculoviruses we created.

The baculoviruses and consequently the strains they are propagated in have likewise evolved. The original strain, DH10MultiBac, contained our engineered virus with the gene deletions we introduced, and a LoxP site on the virus for accepting further functionalities in addition to those entering the standard Tn7 site. By introducing a plasmid for expressing Cre recombinase into the cells harbouring the MultiBac virus, Cre enzyme could be produced, which would then catalyze integration of DNA into the LoxP site on the virus *in vivo*. We have, albeit rarely, used this option, since it is considerably more cumbersome as integrating into the Tn7 site. Eventually, we reengineered this region of the virus and integrated a YFP gene to create EMBacY, the virus which we now are using standardly. EMBacY does no longer allow for accessing a LoxP site in the virus backbone via co-expression of Cre. The Cre reaction however is now much more beneficially used for integrating Donors directly into the Acceptors (see Fig. 3) before the resulting multigene plasmid enters the virus by Tn7 transposition. Expression of YFP has proven to be invaluable for simplifying handling in our hands. With the EMBacY virus, direct feedback, almost in real time, is possible about protein production and virus performance – by measuring the fluorescence, calibrated to a standard and to 1 Mio cells, directly from samples taken from the expression culture.

We developed the MultiBac system out of a simple necessity – we could not obtain the complexes we wanted to study otherwise and would have had to drop those projects. We chose instead to develop MultiBac, and it is gratifying to see now that our method has not only helped us, but many others to carry out their research projects. We are continuously optimizing our reagents and protocols, as our protein complex research requires it, and we will certainly continue to communicate our progress, to the benefit of all.

Figures:

Fig. 1: The MultiBac System 2011.

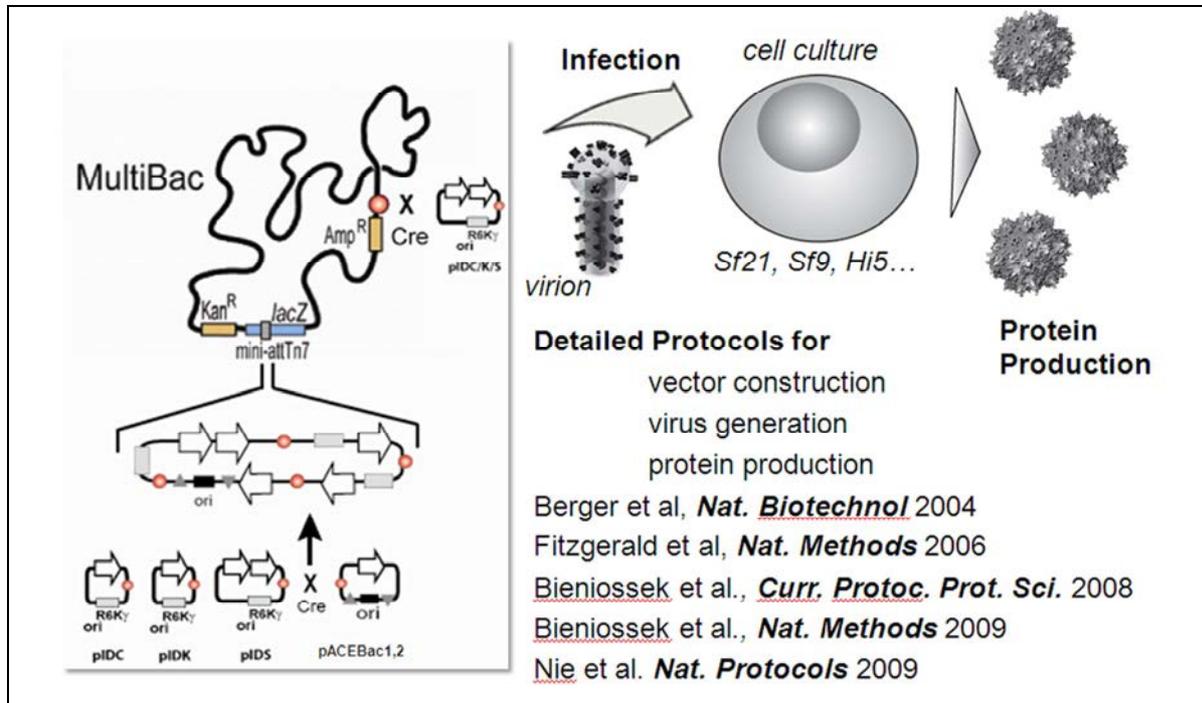


Fig. 2: The MultiBac Protein Production Pipeline at the EMBL Grenoble.

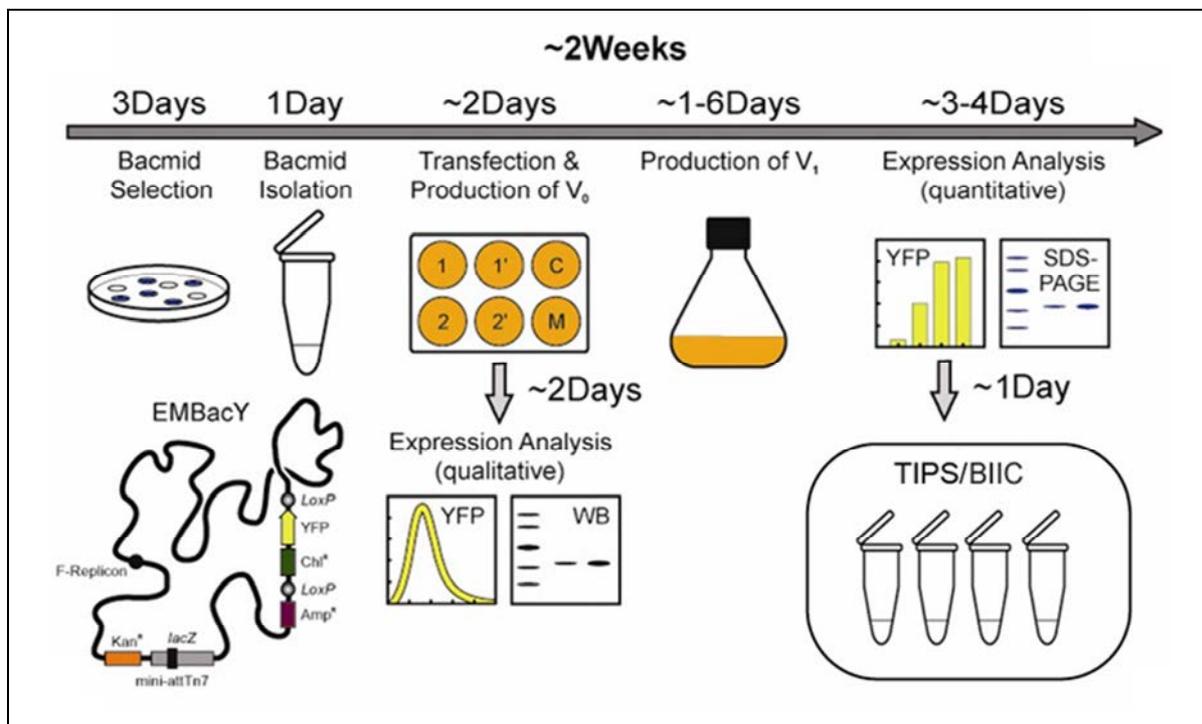
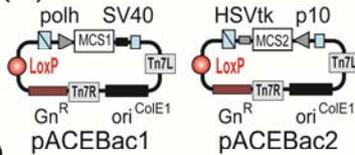


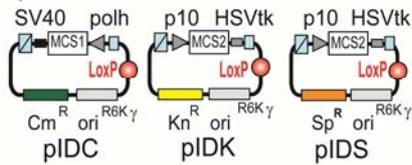
Fig. 3: Multigene plasmid construction by Donor-Acceptor recombineering.

a.

Acceptors (A)

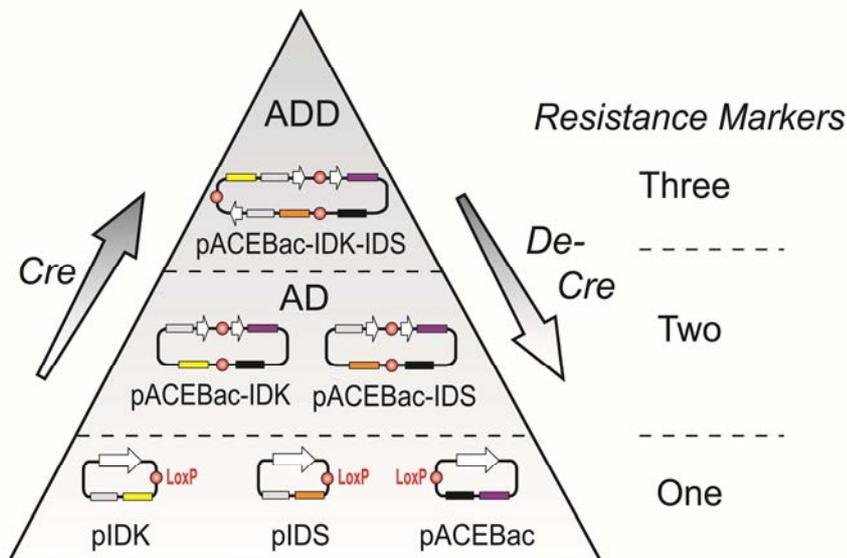


Donors (D)



Expression Cassettes		Multiplication Elements
polh	Promoter sequences	● LoxP repeat seq.
p10	sequences	◻ I-CeuI HE site
MCS1	Sequences for gene integration (i.e. by SLIC)	◻ PI-SceI HE site
MCS2		◻ BstXI (compl. HE)
SV40	polyA term.	
HSVtk	sequences	
ori^{R6Kγ}	Cond. replicon	Ap, Cm,Kn,Sp,Gn: Resistance markers
F	Single-copy replicon	

b.



Reviewer comments:

Reviewed by Anika Altenfeld and Andrea Musacchio
IFOM-IEO Campus, Milan, Italy

- (1) The constructs which are fused must be checked before using them for fusions.
- (2) Overnight incubation is too long, leads to more blue colonies. Incubate for 6 h.
- (3) 2-3 days.
- (4) We observed that 27°C +/- 1°C is OK.
- (5) Cells should divide at least once, but not more than 2 times.
- (6) We thaw cells in hand, to reduce contamination.