# Outline of the Baculovirus Expression System procedure

**Written by: Alma Seitova**

# I. Generation of Recombinant Bacmid DNA

# 1. Preparation of LB agar selective plates for DH10Bac™ transformation

Prepare at advance LB agar plates containing 50 ug/mL kanamycin, 7 ug/mL gentamicin, 10 ug/mL tetracycline, 200 ug/mL Bluo-gal, and 40 ug/mL IPTG to select for DH10Bac™ transformants.

|  |  |  |
| --- | --- | --- |
| Solution | Stock concentration | Storage |
| Gentamicin | 7 mg/ml in water | - 20 °C |
| Kanamycin | 50 mg/ml in water | - 20 °C |
| Tetracycline | 10 mg/ml in 70% ethanol | - 20 °C |
| Bluo-gal | 200 mg/ml in DMSO | - 20 °C |
| IPTG | 40 mg/ml in water | - 20 °C |

Stock solutions dissolved in water should be sterilized by filtration through a 0.22-micron filter. Dispense the solutions into several 1 ml aliquots.

**!**Use of Lennox L (LB) agar instead of Miller's formulation Luria agar plates will reduce color intensity and may reduce the number of colonies. The use of X-gal instead of Bluo-gal will decrease color intensity.

* Weigh 25 g of premixed LB broth (MILLER) and 13 g of Bacto Agar and put into 2 L flask. Bring the volume to 1 L with distilled water and autoclave for 15 -30 minutes at 121°C
* Set a water bath at 50 °C and cool the autoclaved agar solution in the water bath for 40 to 60 minutes until it’s cooled down to 50-550C
* To the cooled solution, add gentamicin to 7 μg/ml, kanamycin to 50 μg/ml, tetracycline to 10 μg/ml, Bluo-gal to 200 μg/ml, and IPTG to 40 μg/ml.
* Mix the agar solution and aliquot 7-10 ml of medium to each 60 mm plate using a 50 ml-pipette. Let the plates sit at room temperature to harden for 2 hours. Invert, wrap and store at 4°C.

**!** Tetracycline and Bluo-gal are light sensitive, so make sure that plates are stored protected from light. Plates containing antibiotics are stable for up to 4 weeks.

**2. Transformation of recombinant plasmid into DH10BAC**

* Calculate the required volume of competent cells (each vial has ~100-110ul).Thaw DH10Bac competent cells on ice, gently spin down, resuspend back by gentle tapping of the bottom of the tube and distribute into PCR strip tubes to be able to use multichannel pipette.
* Dispense 4 ul of the cells into each well of PCR plate or PCR strip tubes, depending on the numbers of targets to be transformed.
* Add 10ul recombinant plasmid DNA (10ul if DNA prep done in High-throughput cloning, in case of regular mini-prep 3-5 should be enough) directly to the competent cells and mix by gently shaking the block. Incubate the mixture on ice for 30 minutes.
* Heat-shock the mixture at 42 °C for 45 seconds. Chill on ice for 2 minutes.
* Dispense 500 μl of S.O.C. medium to each well of 24 or 96 well blocks. Transfer the transformed bacterial suspension into the corresponding well of the block, cover with airpore sheet.
* Place the block in a shaking incubator at 37 °C with medium agitation (205 rpm) for 4-5 hours
* Spread 55 μl of culture evenly over the surface of the LB agar plate. Store the rest of the culture at 4 °C. Incubate the plates at 37 °C until the color of the colonies is discernible (40 to 48 hours).
* Check the total number of colonies on the plate early next morning. If the number is more than 100 or less than 10, spread adjusted volume of culture on another plate and incubate at 37 °C until the color of the colonies is discernible.

Discard the culture when enough white colonies are obtained on the plate.

**!** (Media other than S.O.C. Medium can be used, but the transformation efficiency will be reduced. Expression in Luria Broth reduces transformation efficiency a minimum of two- to three-fold (Invitrogen).

Formulation for 1 Liter of 2x LB media:

Tryptone 20g

Yeast Extract 10g

NaCl 10g

**3. Isolation of Recombinant Bacmid DNA**

3.1(Invitrogen protocol)

**Hints:**  True white colonies tend to be large; therefore, to avoid selecting false positives, choose the largest, most isolated white colonies. Avoid picking colonies that appear gray or are darker in the center as they can contain a mixture of cells with empty bacmid and recombinant bacmid .By holding the plate over a dark and light background it is easier to determine whether a colony is blue or white (whites are more distinguishable against a dark background and blues against a light background).(LifeTech)

* Inoculate a single colony confirmed as having a white phenotype into

3 ml LB medium supplemented with 50 μg/ml kanamycin, 7 μg/ml gentamicin,

and 10 μg/ml tetracycline. Grow at 37°C overnight shaking at 250 rpm.

* Centrifuge the 24 well blocks at 3000RPM for 10 minutes. Decant the supernatant into a suitable container for virkon decontamination. Invert the blocks and tap gently on filter paper. Add 250 µl of Solution 1 to each well.
* Transfer well resuspended cells into 1.5-ml microcentrifuge tube and add

250ul of Solution II, invert gently 5 times and incubate at room temperature for 5 minutes. **Note:** The appearance of the suspension should change from very turbid to almost translucent.

* Add 250ul of Solution3, invert gently 5 times. A thick white precipitate of protein and *E. coli* genomic DNA will form. Place the sample on ice for to 10-15 min.
* Centrifuge for 15-20 min at 14,000 × *g*. at 40C. During the centrifugation, label another microcentrifuge tube and add 0.8 ml absolute isopropanol to it.
* Gently transfer the supernatant to the tube containing isopropanol. Avoid any

white precipitate material. Mix by gently inverting tube a few times and place on

ice for 5 to 10 min. At this stage, the sample can be stored at –20°C overnight.

* Centrifuge the sample for 15 min at 14,000 × *g* at 40C. Remove the supernatant and add 0.7 ml 70% ethanol to each tube. Invert the tube several times to wash the pellet. Centrifuge for 5 min at 14,000 × *g* at room temperature. (Optional: repeat wash)
* Remove as much of the supernatant as possible. **Note:** The pellet may become

dislodged from the bottom of the tube, so watch the pellet when discarding supernatant.

* Air dry the pellet inside of the laminar flow hood for 10 - 15min and dissolve the DNA in 40 μl of filtered TE.
* Recombinant bacmid DNA is greater than 135 kb in size. To avoid shearing the DNA allow the solution to sit in the tube with occasional gentle tapping of the bottom of the tube. The DNA is generally ready for use within 10 min, as long as the pellets are not over dried.
* Store the bacmid DNA at 4°C (see Important below).

Notes:

* You may store your bacmid DNA at –20°C if you avoid frequent freeze/thaw cycles as it decreases the transfection efficiency. To store your purified bacmid DNA at –20°C, aliquot into separate tubes in TE Buffer, pH 8.0 to avoid more than one freeze/thaw cycle and do **not** store in a frost-free freezer. You may also store the purified bacmid DNA for up to 2 weeks at 4°C in TE Buffer, pH 8.0.
* Make sure to use EB Buffer for dissolving DNA, not TE Buffer which has EDTA.

EB Buffer: 10mM Tris-Cl, pH 8.5, Store at RT

TE Buffer: 10mM Tris-Cl, pH 8.0, 1mM EDTA, Store at RT

Solutions: from Millipore

|  |  |  |  |
| --- | --- | --- | --- |
| Fisher | LSKCRS500 | Solution 1 resuspension | 500ml |
| Fisher | LSKCLS500 | Solution 2 lysis | 500ml |
| Fisher | LSKNS0500 | Solution 3 neutralization | 500ml |
| Fisher | LSKP MRN 30 | Rnase A, 30 mg ( in 50% glycerol) | 30mg |

Bacmid solutions from Invitrogen

Solution I 15 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 μg/ml RNase A

Solution II 0.2 N NaOH, 1% SDS

Solution III 3M potassium acetate (pH 5.5)

**Qiagen solutions used by GSK for bacmid DNA extraction**

P1 - 15mM Tris, pH 8.0, 10mM EDTA, 100ug/ml RNase A

P2 - .2N NaOH

P3 - 3.0 Potassium Acetate, pH 5.5

**3. Isolation of Recombinant Bacmid DNA**

3.2 (Invitrogen protocol modified by Alma)

**Hints:**  True white colonies tend to be large; therefore, to avoid selecting false positives, choose the largest, most isolated white colonies. Avoid picking colonies that appear gray or are darker in the center as they can contain a mixture of cells with empty bacmid and recombinant bacmid .By holding the plate over a dark and light background it is easier to determine whether a colony is blue or white (whites are more distinguishable against a dark background and blues against a light background).(LifeTech)

* Inoculate a single colony confirmed as having a white phenotype into

3 ml LB medium supplemented with 50 μg/ml kanamycin, 7 μg/ml gentamicin,

and 10μg/ml tetracycline in 24 well blocks. Grow at 37°C overnight shaking at 250 rpm.

* Centrifuge the 24 well blocks at 3000RPM for 10 minutes. Decant the supernatant into a suitable container for virkon decontamination. Invert the blocks and tap gently on filter paper. Add 250 µl of Solution 1 to each well.
* Seal the blocks with tape pads or any other sealing films and place onto shaking platforms for 5-10 min. Check each well and resuspend if necessary using a 1ml tips.
* Add 250 µl of Solution 2 to each well, seal the blocks and place onto shaking platform for 30sec. and incubate at room temperature for 4 minutes. **Note:** The appearance of the suspension should change from very turbid to almost translucent.
* Add 250ul of Solution3, seal the blocks and place onto shaking platform for 30sec. A thick white precipitate of protein and *E. coli* genomic DNA will form. Place the sample on ice for to 10-15 min.
* Centrifuge for 15-20 min at 3000RPM at 40C. During the centrifugation, label another microcentrifuge tube and add 0.8 ml absolute isopropanol to it.
* Gently transfer the supernatant to the tube containing isopropanol. Avoid any

white precipitate material. Mix by gently inverting tube a few times and place on

ice for 5 to 10 min. At this stage, the sample can be stored at –20°C overnight.

* Centrifuge the sample for 15 min at 14,000 × *g* at 40C. Remove the supernatant and add 0.7 ml 70% ethanol to each tube. Invert the tube several times to wash the pellet. Centrifuge for 5 min at 14,000 × *g* at room temperature. (Optional: repeat wash)
* Remove as much of the supernatant as possible. **Note:** The pellet may become

dislodged from the bottom of the tube, so watch the pellet when discarding supernatant.

* Air dry the pellet inside of the laminar flow hood for 10 - 15min and dissolve the DNA in 45 μl of filtered TE.
* Recombinant bacmid DNA is greater than 135 kb in size. To avoid shearing the DNA allow the solution to sit in the tube with occasional gentle tapping of the bottom of the tube. The DNA is generally ready for use within 10 min, as long as the pellets are not over dried.
* Store the bacmid DNA at 4°C (see Important below).

Notes:

* You may store your bacmid DNA at –20°C if you avoid frequent freeze/thaw cycles as it decreases the transfection efficiency. To store your purified bacmid DNA at –20°C, aliquot into separate tubes in TE Buffer, pH 8.0 to avoid more than one freeze/thaw cycle and do **not** store in a frost-free freezer. You may also store the purified bacmid DNA for up to 2 weeks at 4°C in TE Buffer, pH 8.0.
* Make sure to use EB Buffer for dissolving DNA, not TE Buffer which has EDTA.

EB Buffer: 10mM Tris-Cl, pH 8.5, Store at RT

TE Buffer: 10mM Tris-Cl, pH 8.0, 1mM EDTA, Store at RT

**4. Screening of colonies for the confirmation of transposition**

**by PCR on Bacmid DNA**

Randomly choose and label 2 isolated colonies from each plate. Pick up about half of colony with pipette tip and add to PCR mix.

PCR mix:

Buffer 2 μl

dNTPs 0.4 μl (10 mM each)

Pol. 0.2 μl (NEB Taq)

Fwd primer 0.2 μl (25 uM)

Rev primer 0.2 μl (25 uM)

SDW 16 μl

PCR cycles:

95ºC 2 mins

95ºC 30 sec X25 cycles

55ºC 30 sec

72ºC 1 min/kb

72ºC 1 min

Primers:

pFBOH Fwd CCGGATTATTCATACCGTCCCACCA

pFBOH Rev CTGATTATGATCCTCTAGTACTTCT

Run 10 μl of PCR product on Agarose gel. Primers add about 400 bp to the insert. Plasmids without insert result in a 400 bp band, or no PCR product.

II. Generation of recombinant viral stocks

**1. Transfection of Sf9 cells with recombinant Bacmid DNA/JetPrime**

* Split cells in HyQ-SFX-Insect serum free medium one day in advance to final cells density 1x106 cells/ml. This is to allow the cells to reach mid-log phase.( …it means better do transfections at Tuesday or Thursday)
* Dilute the mid-log phase Sf9 cells to 3x105 cells/ml in HyQ-SFX-Insect medium Seed 0.3 ml (1.5x105 cells) into each well of four 24-well TC plates.
* After pipetting the cells into the plates, mix gently in a side-to-side and
* back-and-forth pattern to ensure an even monolayer. Do not swirl the plates because the cells will cluster into the center of the well.
* Incubate the plate at 27 °C until needed.( ~ one hour)
* Dilute 5ul of JetPrime in 100ul of JetPrime Buffer(JPF) and gently vortex for 10 s
* Dispense 105 μl of the mixture into a sterile 96-Well Microwell Plates
* Transfer 10 μl of recombinant Bacmid DNA into each well and mix by shaking the plate gently.
* Dilute 2ul of JetPrime in 50ul of JetPrime Buffer(JPF) and gently vortex for 10 s
* Mix 6ul of Bacmid DNA with TR diluted in JPB, incubate 10min.
* Overlay dropwise the transfection mix onto the cells in corresponding well of transfection plate and incubate for 5 hours at 270C.
* Immediately gently rock the plates back-and-forth and side-to-side to ensure even distribution of the transfection mixture over monolayer of the cells
* After 5 hours of post- transfection time add 1 ml of HyQ-SFX-Insect serum free medium supplemented with 10% FBS and add antibiotics to 1% final (100 units/ml penicillin and 100 ug/ml streptomycin, P/S)
* Incubate cells in a 27 °C incubator for 72 to 96 hours. Gently mix the culture once a day when is possible
* Look for signs of infection (SIF) for the transfected cells 72 hours post transfection. Collect viruses when cells are well infected, store the P1 viral stocks in dark at 4 0C.

Notes:

A.Virus-infected cells become uniformly rounded and enlarged, with distinct enlarged nuclei. They appear grainy when compared with healthy cells under the phase-contrast inverted microscope. Signs of viral infection are classified as early (within the first 24 hours), late (24–72 hours) and very late (> 72 hours).

* Early: Increased cell diameter. A 25–50% increase in the diameter of the cells

may be observed. Increased size of cell nuclei. The nuclei may appear to "fill" the cells.

* Late: Cessation of cell growth. Cells appear to stop growing when compared to a cell-only control. Granular appearance. Signs of viral budding; vesicular appearance to cells. Viral occlusions. A few cells will contain occlusion bodies, which appear as refractive crystals in the nucleus of the insect cell. Detachment. Cells release from the dish or flask.
* Very Late: Cell lysis. A few cells may fill with occluded virus, die, and burst

leaving signs of clearing in the monolayer.

B. Serum proteins in FBS act as substrates for proteases.

**2. Transfection of Sf9 cells with recombinant Bacmid DNA/Cellfectin**

* Split cells in HyQ-SFX-Insect serum free medium one day in advance to final cells density 1x106 cells/ml. This is to allow the cells to reach mid-log phase.( …it means better do transfections at Tuesday or Thursday)
* Dilute the mid-log phase Sf9 cells to 3x105 cells/ml in HyQ-SFX-Insect medium Seed 0.5 ml (1.5x105 cells) into each well of four 24-well TC plates.
* After pipetting the cells into the plates, mix gently in a side-to-side and
* back-and-forth pattern to ensure an even monolayer. Do not swirl the plates because the cells will cluster into the center of the well.
* Incubate the plate at 27 °C until needed.( ~ one hour)
* Dilute separately 2ul of Cellfectin and 6ul of Bacmid DNA in 50ul of HyQ-SFX-Insect serum free medium and gently vortex for 10s
* Mix together diluted transfection reagent and DNA and incubate for 30min.
* Overlay dropwise the transfection mix onto the cells in corresponding well of transfection plate. Immediately gently rock the plates back-and-forth and side-to-side to ensure even distribution of the transfection mixture over monolayer of the cells
* Incubate for 5 hours at 270C.
* Aspirate HyQ-SFX-Insect serum free medium containing transfection mix from transfection plates, immediately add 1.5 ml of Grace's insect medium supplemented with 10% FBS and P/S
* Incubate cells in a 27 °C incubator for 72 to 96 hours. Gently mix the culture once a day when is possible
* Look for signs of infection for the transfected cells 72 hours post transfection. Collect viruses when cells are well infected and store the P1 viral stocks in dark at 4 0C.

Note: Serum proteins in FBS act as substrates for proteases.

Nunc™ 96-Well Polystyrene Round Bottom Microwell Plates

Cat#262162 or 12565212, Non-Treated

**2. Amplification of viral titer from stage P1 to P3**

2.1 In case of uncertainty for SIF in transfection plates collect P1 virus and amplify viral

titer to stage of P2 in 24 well plates, so you can check carefully the development of

SIF under microscope

* Dilute the mid-log phase Sf9 cells to 3x105 cells/ml in HyQ-SFX with P/S and 2% FBS, seed 1.5ml of the diluted cells into 24 well plates.
* Add dropwise on top of the cells 150ul of P1 recombinant viral stocks, gently swirl a few times and incubate the plate at 27 °C.
* Look for signs of infection (SIF) in 24 hours post infection time. Collect only P2 viruses with SIF and store viral stocks in dark at 40C.
* **!** Immediately (at same day when collecting P2) discard P1 viruses which didn’t have SIF at P2 infection plate to avoid furthest confusions
* Test expression will be done by infecting the cells in 24 well block by P2

2.2 If P1 virus in transfection plate has a good signs of infection proceed to

amplification of the viral titer in 24 well blocks, collect supernatant as P2 and use

pellets for test expression

* Dispense 3ml of Sf-9 cells at a density of 3.5-4mln/ml in HyQ-SFX-Insect serum free medium in into each well of 24-well blocks
* Add 120ul of P2 recombinant virus to corresponding wells to infect suspension culture of cells and cover the blocks with airpore sheet. Incubate at 27oC, with shaking at 245 RPM for 72 hrs.
* After 72 hours post-infection time spray over the 24 well blocks with 70% ethanol, bring into the laminar flow hood and check the cells density and viability in a few wells.
* Seal the plate with a sterile sealing tape and pellet the cells by centrifuging the 24-well blocks at 1500 rpm at 4 °C for 15 minutes
* Spray over the block with 70% ethanol, bring the blocks into the laminar flow hood and collect P2 virus without disturbing the pellets. Place the viral stocks in dark at 40C.
* Wash the cell pellets once with 1 ml of ice cold PBS, invert and tap the blocks firmly on absorbent paper to remove residual PBS
* Resuspend the pellets in 0.5ml of lysis buffer supplemented with protease inhibitors and 0.6% NP-40, and store at -800C for the further test purification.

2.3 Recombinant viral stocks for protein production

* Depending of required volume of production infect 100-200ml of SF9 cells with 100-200ul of P2 respectively at density of cells 3.5-4mln/ml and harvest P3 at cells viability 30-40% (4-5 days)
* For production use 10-15ml of P3 virus for 800ml of SF9 cells at cells density ~ 3.5-4mln/ml and harvest after 72 hours post infection time
* For co-expression infect the cells with 5ml of each of co-expressing targets

! Note: Virus-infected cells have an increased need for oxygen and therefore the

contents of the shake flasks should be shaken at quite high speeds to maximize aeration. The surface area to volume ratio should also be as large as possible for maximum gas exchange – do not overfill flasks!

**IV. High-Throughput Test Expression for intracellular proteins**

1. Assembly of Binding plate:

* Place 4 layers of parafilm on top of the 96 deep well block
* Place a 96 well filter plate on top of the 96 deep well block with seals and push down to secure the tips of the binding plate into the parafilm/plate seal
* Critical to ensure that the top plate won’t leak during incubation
* Use a rubber band to keep the plates together during incubation and centrifugation steps.

2. Test expression procedure

* Place the frozen pellets in a water bath at RT for 5min and to make sure that cell pellets are completely suspended shake the 24-well blocks at 450 rpm for 20 min
* Remove 15ul of total cell lysate into PCR plate with 5ul of SDS PAGE dye, keep at -200C until requested
* Centrifuge the 4x24 well blocks at 3750 RPM for 30 minutes. Transfer the cleared lysates into pre-assembled Binding plate containing 50ul of pre-equilibrated 50% Ni-NTA resin slurry, seal filter plates with special rubber 96 well lid
* Place for 30 min into rotator in cold room the secured Binding blocks for incubation with Ni-NTA resin
* After binding spin down the blocks for 2 min at 1500 RPM and wash 2x with 2ml of Binding Buffer
* Spin down the blocks with washing buffer each time for 5 min at 1500 RPM to make sure to remove all of the residual liquid
* Add 30ul of elution buffer to each well and place the blocks on the top of 96 well microtiter plate containing 10ul of 4x loading dye, incubate for 5 min.
* Spin down the blocks to elute proteins into the 96 well plates at 1000 RPM for 5min. Seal the plate and heat at 98 °C for 3 min. Run the gels.
* Stain the gel with Commassie blue and distain to visualize the protein bands.

Buffers:

Lysis buffer: 25mM Tris pH 8.0, 150-300 mM NaCl, 5% glycerol, 1 X protease

inhibitor cocktail, 0.6 % NP-40, 5% glycerol (v/v), and 2 mM imidazole.

( in old days used only PMSF solution: 0.05 M PMSF and 0.1 M benzamidine)

# Wash buffer: 25mM Tris pH 8.0, 150-300 mM NaCl, 5% glycerol, and 15 mM immidazole;

# Elution bufferfor: 25mM Tris pH 8.0, 150-300 mM NaCl, 5% glycerol, 500 mM imidazole.

|  |  |  |
| --- | --- | --- |
| 1 X protease inhibitor mix | 0.1 mg of Aprotinin, 0.1 mg of Leupeptin, 0.2 mg of Pepstatin A and 0.1 mg of E-64 | Choice of PI depends on type of protein |

CD of cells in Transfection plates:

24 well plate – 0.3mln/ml. total 0.5ml

12 well plate – 0.8mln/ml, total 0.5ml

6 well plate – 1mln/ml, 1ml

CD of cells in Virus amplification plates

24 well plate – 0.3mln/ml. total 1.5ml

12 well plate – 0.5mln/ml, total 2ml

6 well plate – 0.5mln/ml, total 2.5ml