9. Gently pipette 2ml of agarose-overlay into each dish. Incubate at room temperature for 15 minutes or until solidified.
10. When overlay has set, add 1ml of insect cell culture medium to each dish.
11. Place dishes into a sandwich box and incubate at 28°C for 3 days (*Sf*21 cells) or 3-4 days (*Sf*9 cells).
12. Remove liquid overlay and add 1ml of diluted Neutral Red stain (prepare a 5mg/ ml (w/v) stock of neutral red and dilute fresh every time 1: 20 with PBS) to each dish. Incubate for 2 to 4 hours at 28°C. Tip off stain into disinfectant and leave the dishes in the dark to allow plaques to clear for 2-4 hours or overnight.

    If using a LacZ-positive virus, this can be stained with X-gal by adding 1ml of appropriate insect cell medium containing 15µl (2%w/v) X-gal and incubate for 5 hours at 28°C. Plaques will appear blue in colour.
13. Select one set of duplicate dishes with between 10-30 plaques and count them. Determine the average number of plaques for this dilution. To calculate the virus titre use the following calculation:

    Titre of virus (pfu/ml) = average plaque count x dilution factor x 10

    For Example:

    25 plaques (average) on 10^{-6} dilution dishes would give a titre of:

    $25 \times 10^{6} \times 10 = 2.5 \times 10^{8}$pfu/ml.

See website for further details and full downloadable *flash***BAC**™ manuals.

the science of baculovirus expression™

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QUICK START: *flash*BAC™

QUICK START GUIDE to the *flash*BAC™ System

A complete and detailed *flash***BAC**™ manual, for each member of the *flash***BAC**™ family, is available to download from the OET website at www.oetltd.com.

All procedures must be carried out using aseptic technique.

the science of baculovirus expression™

PRODUCTS ARE FOR RESEARCH PURPOSES ONLY, NOT FOR DIAGNOSTIC OR THERAPEUTIC USE

Provided in the kit:

- *flash***BAC**™ DNA
- (Use 100ng [5µl] per co-transfection [20ng/ml])
- Positive-control transfer vector DNA
- (Use 500ng [5µl] per co-transfection [100ng/ml])

To be provided by user:

- 35 mm tissue culture treated dishes seeded with insect cells in a sub-confluent monolayer (*Sf*9 or *Sf*21) [see section 10 of manual for information about insect cells]
- Serum-free insect cell culture medium

If using serum-supplemented medium, you will need medium with and without 10% foetal bovine serum.

- Sterile baculovirus transfer vector DNA containing the gene of interest (500ng per co-transfection)

Any vector designed for double-crossover, homologous recombination with baculovirus DNA at the polyhedrin locus is suitable [see website for more details]. The DNA must be sterile and must be of a quality suitable for transfection into cells

- Transfection reagent. Reagents tested and found to be successful are Lipofectin® (Invitrogen), FuGENE 6 (Roche), GeneJuice® (Novagen), Tfx-20™ (Promega)
- Incubator set at 28°C
- 1% Virkon (Amtec), or other suitable disinfectant
- Inverted phase-contrast microscope
- Plastic box to house dishes in the incubator
- Sterile pipettes, bijoux or similar

Note that plasticware used to prepare the transfection mixture must be made from polystyrene and not from polypropylene.

1.1 Production of recombinant viruses using the *flash***BAC**™ system:

1. Prepare one 35mm dish of insect cells (*Sf*9 or *Sf*21) for each co-transfection.
2. Pipette 1 ml serum-free, antibiotic-free medium into a sterile, disposable polystyrene container for each co-transfection
3. Add appropriate volume of transfection reagent (e.g. 5µl Lipofectin®) and mix.
4. Add 100ng *flash***BAC**™ DNA (5µl from the kit) and either 500ng transfer vector DNA containing the gene of interest or 5µl of control DNA. Mix gently by agitation or vortexing.
5. Incubate at room temperature for 15-30 minutes.
6. Before the end of the incubation period, remove the culture medium from the dishes. If the cells are in serum-supplemented media, then wash twice with 1ml of serum-free media. This washing step is not necessary with cells in serum-free media.
7. As soon as the medium has been removed, add the 1ml of DNA + Liposome complex drop-wise into the centre of each dish. Incubate in a sandwich box overnight or for at least five hours at 28°C.
8. After this time, add 1ml of appropriate insect cell culture medium to each dish. If using serum-supplemented medium, add serum at this stage.
9. Further incubate at 28°C for five days.
10. Harvest the medium containing the recombinant virus into a sterile bijou by aseptic technique and store at 4°C in the dark (this is your seed stock of recombinant virus).

1.2 Amplification of recombinant *flash***BAC**™ virus:

1. Prepare a 100-200ml culture of *Sf*9 or *Sf*21 cells at appropriate cell density (in log growth phase).
2. Add 0.5ml of the recombinant virus seed stock (See 1.1) to the cell culture and incubate with shaking or stirring (as appropriate) until the cells are well infected, normally 4-5 days.
3. Remove the cell from the culture medium by centrifugation at 3000rpm at 4°C for 15 minutes.
4. Decant aseptically and store the culture medium at 4°C in the dark. At this point the virus can be stored at 4°C for 6-12 months or -70°C for longer periods of time, although the virus may need to be re-titred before use. Do not store the virus at -20°C.
5. It is strongly recommended to plaque assay the recombinant virus before use to determine the titre (See 1.3) or use the *baculo***QUANT**™ virus titration kit (see website for details).

1.3 Plaque assay to determine titre of recombinant virus:

1. Prepare ten 35mm diameter dishes with *Sf*21 cells (1.4 x 10^6 cells/dish) or *Sf*9 cells (0.9 x 10^6cells/dish) and leave at room temperature for 1 hour.
2. During the incubation time, prepare serial dilutions (1 in 10; 50µl of virus and 450µl medium is recommended) of the virus to be titred from 10^{-1} to 10^{-7}.
3. Check that the cells have formed an even sub-confluent monolayer.
4. Remove the cell culture medium from the dishes into 1% Virkon or other disinfectant.
5. Add 100µl of virus dilutions from 10^{-4} to 10^{-7} to duplicate dishes in a drop-wise manner to the centre of each dish using a sterile pipette for each. Include 2 dishes for mock-infected controls, where 100µl of appropriate insect medium is added.
6. Incubate dishes at room temperature for 1 hour (no longer) on a level surface.
7. About 15 minutes prior to the end of the incubation period, prepare the LGT overlay. Melt 1 x 10ml of prepared and solidified 2% (w/v) LGT agarose in a microwave or boiling water-bath (Ensure that appropriate safety precautions are taken.) Cool to hand hot (50°C) and add an equal volume (10ml) of appropriate insect cell culture medium (with 1% antibiotics if required added to media beforehand). Mix thoroughly, avoiding air bubbles.
8. Remove the virus inoculum from each dish into 1% Virkon or other disinfectant.