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SfC1B5 cells

A complete laboratory guide to insect cell culture

2023-2024

The SfC1B5 cells have also been prepared as a GMP-compliant Master Cell Bank and Working Cell Bank. Further details are available on request

Oxford Expression Technologies Ltd
Bioinnovation Hub, Gipsy Lane, Oxford. OX3 0BP
t: +44 (0) 1865 483236 f: +44 (0) 1865 483250
e:info@oetltd.com w: www.oetltd.com

Insect cell culture guide for SfC1B5 cells

On delivery of your cells from OET, you will find 2 vials of frozen cells (each with ~2 x 10^7 cells) that should immediately be stored in liquid nitrogen. For recovery of the cells, please follow the guidelines stated in Section 5. If you have any problems recovering the first vial of cells, please contact OET (info@oetltd.com) before attempting to recover the cells from the second vial.

1.0 Insect Cell Culture

This manual provides a guide to the growth, maintenance and experimental use of SfC1B5 insect cell cultures. It is extremely important that the insect cells used for the production and analysis of recombinant baculoviruses are in excellent condition with very high viability. The following protocols provide tried and trusted methods used within the labs at OET and by scientists with over thirty-years' experience of working with insect cell cultures and baculoviruses. We hope it is useful to both beginners and more experienced researchers. **It is also important to note that the SfC1B5 cells have different characteristics, growth rates and seeding densities compared to Sf9 or Sf21 cells, so please read on before starting to use your cells.**

2.0 Introduction to the SfC1B5 cell line

SfC1B5 cells are a clonal isolate from Sf21 cells, which were originally derived from the pupal ovarian cells of *Spodoptera frugiperda* (Fall armyworm) (Vaughn *et al*., 1977). The cells have been expanded in animal-free media (ESF-AF, available from OET) and a Research Bank prepared, from which you have been sent 2 vials under MTA.

The SfC1B5 cells have also been prepared as a GMP-compliant Master Cell Bank and Working Cell Bank. Further details are available on request (info@oetltd.com).

SfC1B5 cells (Table 1, Figure 1) can be used as a direct replacement for Sf9 cells in applications including virus amplification and protein production (Section 8). A typical SfC1B5 growth curve is shown in Figure 2. Like Sf9 cells, they stick firmly to cell culture dishes and so we recommend passaging the cells as suspension shake cultures (see Section 7) but monolayers can be used for transfections to make recombinant viruses and expression testing, for example (see Section 8).

Table 1. SfC1B5 cell characteristics

Cell Line	Appearance	Expression	Doubling Time	Uses	Media
SfC1B5	Spherical, larger and slightly more irregular in size than *Sf9* cells	Expression is shown to be similar to that achieved with Sf9 cells	~30 hours	Monolayer or Suspension cultures Amplification of viruses Production of proteins	ESF-AF™ available from OET, Cat# 500400-500404)

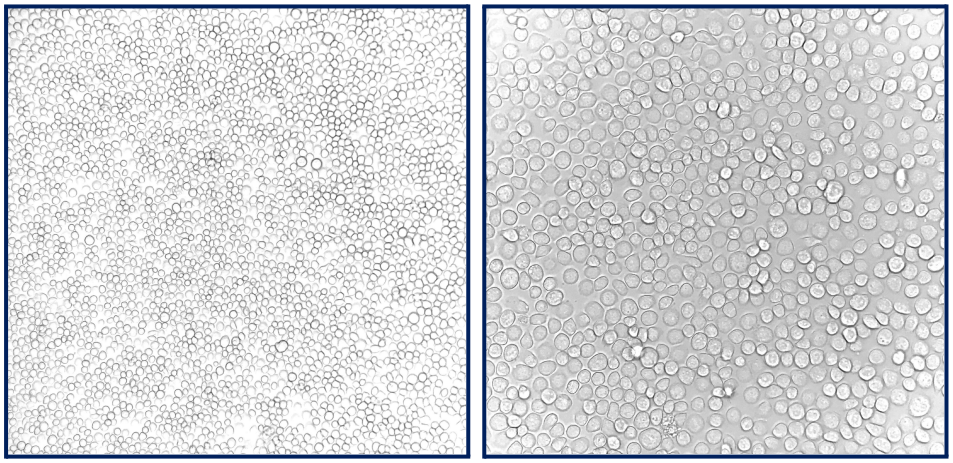


Figure 1.
Bright-field images of SfC1B5 cells taken from a mid-log phase shake culture (placed in a 35 mm dish for imaging purposes). Cells were imaged at x 100 (left panel) and x400 (right panel) using an Echo Revolve microscope.

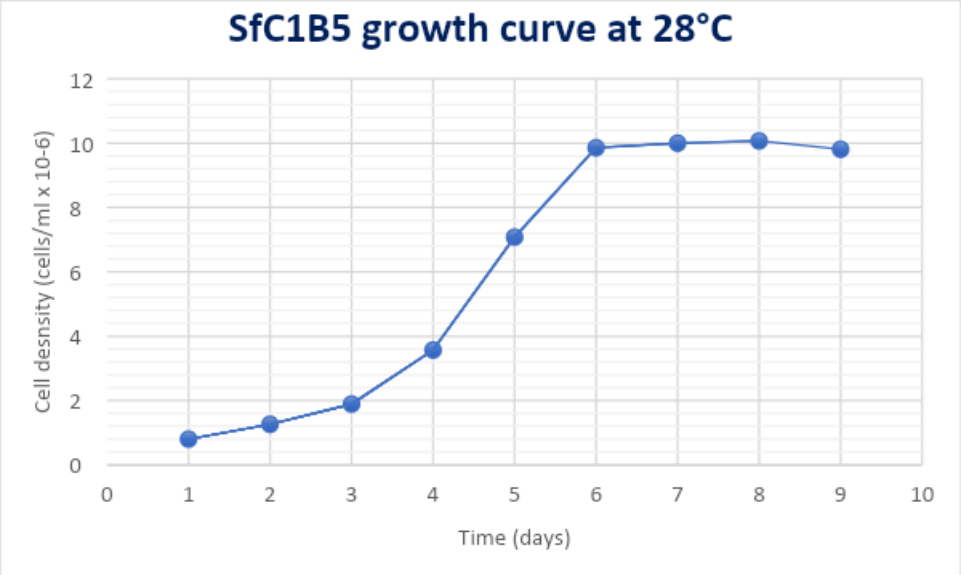
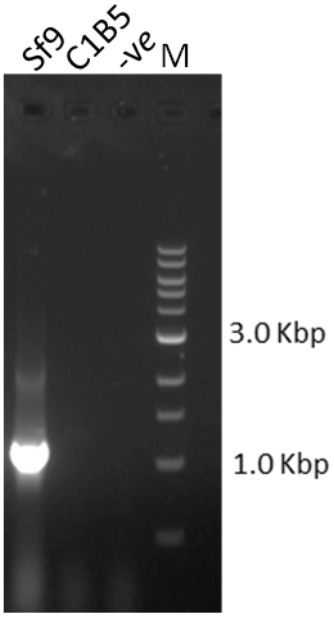


Figure 2.
SfC1B5 cells were seeded at 0.8 x 10^6 cells/ml in 50 ml ESF-AF medium in a 250 ml shake flask at 28°C (on a shaking platform at 120 rpm with 19 mm throw). The culture reaches stationary phase at around 1 x 10^7 cells per ml. Cells should be subcultured before they reach this stage.



The SfC1B5 cell line is free from the rhabdovirus that is found in Sf9 and Sf21 cell lines (Ma *et al.*, 2014), which has advantages for the clinical manufacture of biologicals under GMP conditions. The SfC1B5 cells have been extensively tested and on all occasions have been shown to be negative for the rhabdovirus by PCR (Figure 3).

Random vials from OET's research bank of SfC1B5 cells were selected and tested for sterility, mycoplasma and rhabdovirus. All samples were negative for each test.

Figure 3.
Total RNA was extracted Sf9 and SfC1B5 cells and analysed using RT-PCR with Sf rhabdovirus-specific primers. A negative (water-only; -ve) control was included. M= molecular size markers.

SfC1B5 cells can be used for amplification of high titre recombinant baculoviruses, in the same way as Sf9 cells. Cells should be infected with a low multiplicity of infection (MOI = 0.1 pfu/cell) when the cells are at a density of 0.5 to 1 x 10^6/ml as described in section 8. At OET, we regularly obtain recombinant virus titres between 1-3 x 10^8 pfu/ml (as judged by plaque-assay or QPCR e.g. using OET's BaculoQUANT™).

For protein production in shake flasks, we find we get maximum yields when cells are at a density of c.1.3 x 10^6 cells/ml when they are infected with recombinant virus at high MOI (5+ pfu/cell) – see Section 8. However, this varies considerably depending on the target protein and we recommend you determine optimal conditions of MOI and time to harvest empirically. Sometimes a low MOI (less than 1 pfu/cell) can provide the best yield of protein.

In general, SfC1B5 cells should be used at slightly lower densities than Sf9 cells and whilst we provide indicative cell densities in our protocols (Section 8), every lab counts cells in a slightly different way, so we strongly recommend optimising cell growth conditions, virus amplification and protein production protocols in your own lab to achieve the best results with these cells.

1. Vaughn, J. L., Goodwin, R. H., Tompkins, G. J. & McCawley, P. (1977). *In Vitro* 13, 213-217.
2. *Sf*9 was cloned by G. E. Smith and C. L. Cherry from the parent line, IPLB-SF 21 AE, by Vaughn *et al*., in 1977[1].
3. Ma, H., Galvin, T. A., Glasner D. R., Shaheduzzaman, S,. Khan, A. S. (2014) *Journal of Virology* 88, 6576-6585.

3.0 General Requirements for Insect Cell Culture

Insect cells have a relatively high dissolved oxygen content (DOC) requirement, particularly when infected with baculovirus. Maintaining the appropriate DOC is important for cell growth and virus replication, and this can be achieved in shake, spinner and tissue culture flasks by using vented lids or non-vented lids kept loose. Most insect cells can be cultivated over a temperature range from 25-30°C. The optimal temperature for cell growth and infection for insect cells is considered to be 27-28°C. Insect cells can also be cultured at room temperature (about 20-22°C) when a slower growth rate is required. Virus infection is usually carried out at 27-28°C. **When recovering SfC1B5 cells from frozen stocks, use incubators set at 28°C.**

To ensure sterility and longevity of your cells, all techniques must be carried out using a designated cell culture lab coat, gloves (elbow length preferred) and following good aseptic techniques. This can be ensured by working either in a Class II safety cabinet or tissue culture laminar flow cabinet, where appropriate, and using 70% industrial methylated spirits (IMS) or isopropanol for disinfection of surfaces.

Insect cells are grown in a phosphate-buffered medium and must never be cultured in a standard CO_2 incubator with the high humidity that is used for mammalian cells. **SfC1B5 cells must be cultured in a 'dry' incubator or warm room with just normal room levels of humidity**. Do not use a CO_2 incubator with the 'gas turned off' as gas seepage and high humidity will adversely affect cell growth.

4.0 Equipment and reagents

In this protocol we provide a detailed set of methods for successfully establishing and maintaining SfC1B5 cells in shake cultures starting from a frozen vial. To follow these protocols a list of equipment and reagents required is provided in Table 2.

When working with liquid nitrogen, chemicals and cells, always wear the correct personal protective equipment (PPE). For more information, please consult the appropriate safety data sheets (SDS) and your own risk assessments. Dispose of plasticware in autoclavable waste and dispense waste liquids into Virkon® or similar.

To avoid contamination of cultures, aseptic techniques must be used at all times.

Table 2. Equipment and reagents required for cell culture and experimental work

ITEM	Thawing cells	Freezing cells	Cell Culture	Experimental work*
Equipment				
Class II/Laminar Flow Hood	+	+	+	+
Shaking platform	+	+	+	+
Haemocytometer/cell counter	+	+	+	+
Optical microscope	+	+	+	+
Incubator (28°C)	+	+	+	+
Benchtop Centrifuge		+		+
Floor standing Centrifuge		+		+
Water Bath (30°C)	+			
Pipette Aids (2 µl/ 20 µl/ 200 µL/ 1000 µL)	+	+		+
Freezing chamber (e.g. Mr Frosty® by Nalgene® or CoolCell® by Brooks Life Sciences)		+		
Storage container for cells in liquid nitrogen and appropriate PPE	+	+		
Reagents				
Trypan blue (e.g. Sigma-Aldrich)	+	+	+	
Cell culture media (ESF-AF™ available from OET, Cat# 500400-500404)	+	+	+	+

Virkon®	+	+	+	+
Isopropanol or IMS (70%)			+	
Freezing mix: 45% conditioned growth medium, 45% fresh medium, 10% DMSO (cell culture grade)		+		
Consumables				
Shake culture flasks (125ml – 5L) (with vented caps)	+	+	+	+
T-flasks for monolayer culture back-ups (T25/T75) -vented type	+		+	
Sterile Bijoux (7ml)	+	+	+	+
Sterile Tube (15-50 ml)	+	+	+	+
Cryovials (1.8 ml)		+		
Serological Pipettes (1 ml/12ml/25ml/50ml)	+	+	+	+
Centrifuge Bottle		+		+
Pipette Tips (10 µl/ 200 µl/ 1000 µl)				+
PETG Bottle for virus storage				+
1.5 and 2ml microcentrifuge tubes				+
Cell culture dishes (35mm/ 6 well/12 well plates)				+
Neutral Red stain (for plaque-assays)				+
Low temperature gelling (Sea-plaque®) agarose for plaque-assays				+
Phosphate-buffered saline (PBS)				+
*baculo*QUANT™ from OET to titrate virus by QPCR				+
Plastic Box				+

*This is an indication of what might be required; however, it is dependent on the experimental work being carried out.

5.0 Recovery of cells – suspension culture method

We recommend reading through this section before recovering your vial of cells from liquid nitrogen storage. If you prefer to recover cells into monolayer culture before seeding shake cultures – see Protocol 5.2.

Following recovery, passage the SfC1B5 cells 4-6 times (Section 7) before expanding to prepare your own cell bank (Section 6) or for experimental work (Section 8).

Do not culture the SfC1B5 cells in a hood at the same time as Sf9 or Sf21 cells to avoid the risk of infecting the cells with the rhabdovirus that can be found in the culture medium of these cell lines.

Table 3 shows typical total and viable cell counts after recovering a vial of SfC1B5 cells and shows how the culture can be expanded over 17 days to provide enough cells to prepare a 100 vial cell bank of your own as well as cells for continued culture and experimental use.

The results in Table 3 were obtained by following the protocol described in 5.1, by an independent laboratory.

Table 3. Typical cell counts after recovery from a frozen vial (all in ESF-AF medium)

Day	Stage	Culture size	Total number of cells	Cell density cells/ml	Viable cell density cells/ml	% Viability
0	Recovery	10 ml (1 ml cells from cryovial + 9 ml medium)	1.94×10^7	1.94×10^6/ml	1.58×10^6/ml	82%
0	Initiation	125 ml flask (15 ml)	1.5×10^7	1×10^6/ml	-	-
1	Viability check (optional)	125 ml flask (15 ml)	1.34×10^7	0.92×10^6/ml	0.79×10^6/ml	85%
4	Cell count	125 ml flask (15 ml)	2.27×10^7	1.57×10^6/ml	1.57×10^6/ml	93%
5	Cell count	125 ml flask (15 ml)	3.59×10^7	2.87×10^6/ml	2.73×10^6/ml	95%
5	Seed P+1 & expansion	250 ml flask (50 ml)	4.0×10^7	0.8×10^6/ml	-	-
8	Cell count	250 ml flask (50 ml)	7.35×10^7	1.47×10^6/ml	1.46×10^6/ml	99%
8	Seed P+2 & expansion	1 L flask (225 ml)	1.8×10^8	0.8×10^6/ml	-	-
11	Cell count	1 L flask (225 ml)	5.9×10^8	3.26×10^6/ml	3.1×10^6/ml	95%
11	Seed P+3 & expansion (3 flasks)	3 x 1 L flask (225 ml x 3)	5.4×10^8	0.8×10^6/ml	-	-
14	Cell count	3 x 1 L flask (225 ml x 3)	1.98×10^9	2.88×10^6/ml	2.85×10^6/ml	99%
14	Seed P+4 & expansion	4 x 1 L flask (225 ml x 4)	7.2×10^8	0.8×10^6/ml	-	-

	(4 flasks)					
17	Cell count	4 x 1 L flask (225 ml x 4)	2.45 x 10^9	2.72 x 10^6/ml	2.7 x 10^6/ml	99%
17	Cell Bank	100 vials @ 1 ml	2 x 10^9	2.0 x 10^7/ml	-	-
17	Seed P+5 (1 flask)	1 x 1 L flask (225 ml)	1.8 x 10^8	0.8 x 10^6/ml	-	-
And continue passaging cells in this way – expanding the number of flasks to produce cells for experimental use as required.						

5.1 Protocol for recovery of cells – suspension culture method

We use this protocol to recover cells from storage in liquid nitrogen, however, some researchers prefer to recover cells into a monolayer culture at first and then seed a shake flask – if you prefer to do this, see Section 5.2. If you are inexperienced, protocol 5.2 may be preferred.

Step	Quick Steps	More detailed protocol and guidance notes
5.1	Pre-warm 9 ml ESF-AF medium.	Prepare a clean water bath at 30°C. Prepare 9ml pre-warmed ESF-AF medium in a sterile plastic container.
5.2	Thaw SfC1B5 cells taken from liquid nitrogen storage.	Using the appropriate PPE, carefully remove a vial of the SfC1B5 cells from liquid nitrogen and immediately place in the water bath until only a small piece of ice is visible. A microcentrifuge float is suitable for this purpose. This process normally only takes a few minutes. The thawing temperature can vary +/-2°C of the optimum temperature of 30°C.
5.3	Sterilise the outside of the vial and transfer to a Class II hood.	Rinse or mist the outside of the vials with 70% IMS to sterilise and dry the vial with tissue. Transfer the vial into a Class II hood.
5.4	Dilute cells 1:10 in ESF-AF medium.	Dispense 9 ml of pre-warmed ESF-AF medium into a sterile tube (25mls). Using a 1ml serological pipette, transfer the thawed cells (1 ml) to 9 ml of pre-warmed ESF-AF medium in a sterile tube. Gently mix to evenly distribute the cells.
5.5	Count cells and check viability.	Take a small aliquot of the diluted cells and calculate the cell count and viability using a haemocytometer or cell counter. Take two independent readings and average the two counts. The cell count will usually be between 1.5 x 10^6- 2.5 x 10^6 cells/ml and the viability well above 70%. Note: the original cell density should be ~2 x 10^7 cells/ml as indicated on the vial provided. For further information on cell counting see Section 7.
5.6	Seed a 125 ml shake flask to give a final cell density of **1.0 x 10^6** cells/ml in at least 15 ml ESF-AF medium	Based on the cell count, calculate the volume of additional ESF-AF medium required to obtain a cell concentration of **1.0 x 10^6 cells/ml**. Using a serological pipette, add the diluted cells and medium to a 125 ml shake culture flask. The volume of this initial or starter culture should be at least 15ml.

		Optional: determine the cell count immediately after setting up the cultures to confirm the seeded density.
5.7	Incubate the culture on a shaking platform **at 28°C** and incubate for 3-5 days (over a weekend is ideal)	Place the 125ml shake flask on a rotating platform in an incubator set **at 28°C** and leave for 3-5 days (over a weekend is convenient). For a 125 ml shake flask, we rotate at 120 rpm (19 mm throw). We recommend leaving the cells for 3-5 days to recover as they will be in lag-phase during the first few days. During this time, it is normal for the viable cell count to drop and then the cells start to recover and proliferate after c.72 hours. Best not to disturb them during this phase!
5.8	Seed a new 25/50 ml culture (250 ml flask) at a seeding density of **0.8 x 10^6** cells/ml.	Post 72hr, the cells will usually enter log-phase, proliferate and increase their number exponentially. From this point onwards, the cells can be passaged into fresh 25/50ml cultures at a seeding density of **0.8 x 10^6 cells/ml** in a 250 ml flask. See Section 7 for further information on seeding cells for routine passaging and experimental work. Sometimes a 'tide-mark' appears around the rim of the new culture. This is normal and can be removed either by shaking the cells at increased rpm (to 140) for 1 hour prior to harvest or by swirling the flask by hand. This does not happen with established cultures.
5.9	Gradually expand the culture to prepare sufficient cells for your own cell bank via storage of cells in liquid nitrogen (Section 6), or experimental work (Section 8).	Cultures need to be passaged @ two times/week until the typical phenotypic characteristics of SfC1B5 cells are observed (Tables 1, 3) and the cultures are routinely greater than 95% viable (98-99% is normal). This normally takes between 4 to 6 passages. During the early passages SfC1B5 cells may exhibit granulation with enlarged cells, however, after a few passages they should look as described in Table 1 and Figure 1. Once the cell line has been established, the cultures can be expanded to prepare your own cell bank via storage of cells in liquid nitrogen (Section 6) or used for experimental work (Section 8). Table 3 provides an example of an expansion regime that builds sufficient cells for a 100 vial cell bank using multiple 1 L flasks each with 225 ml culture.

5.2 Protocol for recovery of cells – monolayer culture method

Step	Quick Steps	More detailed protocol and guidance notes
5.1	Pre-warm ESF-AF medium.	Prepare a clean water bath at 30°C. Pre-warm ESF-AF medium in a sterile plastic container.
5.2	Thaw SfC1B5 cells taken from liquid nitrogen storage.	Using the appropriate PPE, carefully remove a vial of the SfC1B5 cells from liquid nitrogen and immediately place in the water bath until only a small piece of ice is visible. A microcentrifuge float is suitable for this purpose. This process normally only takes a few minutes. The thawing temperature can vary +/-2°C of the optimum temperature of 30°C.
5.3	Sterilise the outside of the vial and transfer to a Class II hood.	Rinse or mist the outside of the vials with 70% IMS to sterilise and dry the vial with tissue. Transfer the vial into a Class II hood.
5.4	Dilute cells 1:10 in ESF-AF medium.	Dispense 9 ml of pre-warmed ESF-AF medium into a sterile tube (25mls). Using a 1 ml serological pipette, transfer the thawed cells (1 ml) to the medium. Gently mix to evenly distribute the cells.
5.5	Count cells and check viability.	Take a small aliquot of the diluted cells and calculate the cell count and viability using a haemocytometer or cell counter. Take two independent readings and average the two counts. The cell count will usually be between 1.5 x 10^6- 2.5 x 10^6 cells/ml and the viability well above 70%. Note: the original cell density should be ~2 x 10^7 cells/ml as indicated on the vial provided. For further information on cell counting see Section 7.
5.6	Dispense cells between two T75 cm^2 flasks (vented) and increase volume to 12 ml/flask. Incubate **at 28°C** for 5-12 h. Remove medium and replace with 12 ml fresh ESF-AF medium.	Dispense cells into 2 x T 75cm^2 flasks (vented) and increase volume to 12 ml with additional medium. You should have about 10 x 10^6 cells/flask, although this may vary slightly depending on your cell count. Ensure cells are even spread out over the surface of the flask. With non-vented flasks, the lid should be left slightly loose to allow gas exchange. Incubate cells **at 28°C** for between 5- 12h (or overnight). Then remove all the medium and replace with 12 ml fresh ESF-AF. This removes any dead cells from the culture. The live cells should be stuck down firmly at this stage.
5.7	Continue the incubation **at 28°C** until the cells are very confluent (up to 7 days or longer).	Continue the incubation at 28°C until the cells are very confluent. This may take several days (7 or more). **Monitor the volume of medium in the flask** and if it falls by more than 10% remove half of it and add additional medium to a total volume of 12 ml. **This is important.**

5.8	Harvest the 'very' confluent cells by tapping the flasks sharply on the bench a few times; do not scrape the cells off. Place the cells in a tube to count and seed a shake flask (see 5.9). Add 12 ml fresh medium to the original T-flask (which will have cells left) as a back-up flask and incubate at 28°C.		Harvesting the cells requires that they are very thick to permit detachment from the surface of the flask (sub-confluent or just confluent cells stick like glue!). This is achieved by sharply tapping the flask on its narrow end on a solid surface (e.g. laboratory bench). Continue tapping until the majority of cells are detached – it is impossible to remove them all. **Note**: Do not use a plastic scraper to remove the cells as this will severely reduce their viability. Remove the detached cells from the flask with a pipette and place in a suitable plastic tube. Immediately, add 12ml fresh ESF-AF medium to the old flask. This can be incubated further and serve as a reserve culture.
5.9	Count the cells and determine viability. Seed a 125 ml shake flask to give a final cell density of **1.0 x 10^6** cells/ml in 15-25 ml ESF-AF medium		Count the cells harvested in 5.8. Determine viability (see Section 7 for more details). We would normally expect cells to be >90% viable at this stage. Dilute the cells to 1 x 10^6 cells/ml with ESF-AF medium to give a total volume between 15-25 ml and dispense to a 125 ml shake culture flask.
5.10	Incubate the culture on a shaking platform at 28°C and incubate for 2-3 days.		Place the 125 ml shake flask on a rotating platform in an incubator set at 28°C and leave for 2-3 days. For a 125 ml shake flask, we rotate at 120 rpm (19 mm throw).
5.11	Seed a new 25/50 ml culture (250 ml flask) at a seeding density of **0.8 x 10^6** cells/ml.		After 48-72h, the cells will usually enter log-phase, proliferate and increase their number exponentially. The cells will grow to 10^7 cells/ml (Figure 2) but they should be passaged before they reach this density. From this point onwards, the cells can be passaged into fresh 25/50ml cultures at a seeding density of **0.8 x 10^6 cells/ml** in a 250 ml flask. See Section 7 for further information on seeding cells for routine passaging and experimental work. Sometimes a 'tide-mark' appears around the rim of the culture. This is normal and can be removed either by shaking the cells at increased rpm (to 140) for 1h prior to harvest or by swirling the flask by hand.
5.12	Gradually expand the culture to prepare sufficient cells for your own cell bank via storage of cells in liquid		Cultures need to be passaged about two times/week until the typical phenotypic characteristics of SfC1B5 cells are observed (Table 1) and the cultures are routinely greater than 95% viable (98-99% is normal).

	nitrogen (Section 6), or experimental work (Section 8).	This normally takes between 4 to 6 passages. During the early passages SfC1B5 cells may exhibit granulation with enlarged cells, however, after a few passages they should look as described in Table 1 and Figure 1. Once the cell line has been established, the cultures can be expanded to prepare your own cell bank via storage of cells in liquid nitrogen (Section 6) or used for experimental work (Section 8).

6.0 Liquid nitrogen storage of SfC1B5 cells

Once the SfC1B5 cell line has been established in suspension culture for 4-6 passages, stocks should be expanded to provide sufficient numbers to be frozen in ampoules in liquid nitrogen as a Cell Bank (see Table 3). This enables new cultures to be established when required, for example, after 30 passages or if the culture fails for some reason. It is important to freeze cells at as low a passage number as possible and to ensure that cells are taken from a mid-log phase culture that has high (>95%) viability. There are many variations in the protocols published for the freezing down of insect cells. The following is one that we find works well.

6.1 Protocol for liquid nitrogen storage of SfC1B5 cells

Step	Quick Steps	More detailed protocol and guidance notes
6.1	Harvest (>95% viable) cells from a log phase culture.	For storage of SfC1B5 cells in liquid nitrogen, harvest healthy cells (>95% viability – 98/99% should be aimed for) from a log phase culture (mid log phase, 4 – 6 x 10^6 cells/ml). Try to use cells from a low passage number. Make sure you have enough cells to freeze cells at ~2 x 10^7 cells/ml for the desired number of vials.
6.2	Label the cryovials and place them on ice.	Use sterile cryovials that will hold a 1 ml sample e.g. 1.8 ml vials.
6.4	Count cells and check viability.	Take an aliquot of the cells and calculate the cell count and viability using a haemocytometer or cell counter. Take two independent readings and average the two counts. Make sure the cells are looking healthy (Figure 1) and the viability is >95%. For further information on cell counting see Section 7.
6.5	Pellet the cells and resuspend in **freezing mixture** to give each vial a cell density of 2 x 10^7 cells/ml. Dispense 1 ml aliquots into the cryovials on ice.	Pellet the required number of cells very gently at 500 rpm for 5 minutes to form a soft pellet. Remove the cell culture medium and retain it as 'conditioned medium'. Use the retained conditioned growth medium to prepare the freezing mixture as follows: **Freezing mixture** 45% conditioned growth medium 45% fresh medium 10% DMSO (cell culture grade)

		Resuspend the required number of cells in the freezing mixture (to a density of 2 x 10^7 cells/ml) by very gently pipetting up and place 1 ml aliquots into cryovials. Once DMSO has been added, the cells must be cooled immediately to avoid damage
6.5	Place the vials in a freezing chamber and follow the manufacturer's instructions. Transfer vials to liquid nitrogen storage.	Using appropriate PPE, place the vials in a freezing chamber (e.g. Mr Frosty® by Nalgene® or CoolCell® by Brooks Life Sciences) and follow the manufacturer's instructions before transferring to liquid nitrogen. This normally involves leaving the cells at -80°C overnight. Set up a logbook/e-record system for the cells in storage.
6.6	Check cells retrieved from liquid nitrogen have a viability of >80%.	After a few days, retrieve one or two vials to ensure that the freezing process has been successful. Follow Section 5.1 or 5.2 for revival of cells from liquid nitrogen storage. Keep a logbook/e-record of where and when each cell line is frozen, and when vials are recovered

7.0 Routine passaging of SfC1B5 cells

Sub-culturing, or passaging, of cells allows them to be maintained within log phase, preventing them from entering their stationary phase (see Figure 2), and ensuring optimal viability for experimental use.

It is essential to monitor cells under an inverted microscope prior to and after passaging them. This is to check for seeding density, confluency (if seeding in dishes), any signs of poor health or overgrowth. Signs of poor health include grainy cells, 'sausage' shaped cells, longer doubling time, failure to reach confluence or high cell densities.

We do not routinely use antibiotics or antifungals for any routine cell culture, as they can mask low-levels of contamination that may affect experimental use of the cells. It is better to know that the cells are contaminated, discard the culture and return to a back-up culture or fresh vial from the cell bank.

Do not culture the SfC1B5 cells in a hood at the same time as Sf9 or Sf21 cells to avoid the risk of infecting the cells with the rhabdovirus that can be found in the culture medium of these cell lines.

7.1 Protocol for routine passaging of SfC1B5 cells

Step	Quick Steps	More detailed protocol and guidance notes
7.1	Pre-warm ESF-AF medium.	Pre-warm ESF-AF medium prior to use and turn on Class 2 hood or Laminar flow hood. Spray the hood, media and any materials being used with 70% IMS to sterilise. It is good practice to spray all materials and gloved hands when entering the Class II/Laminar flow hood.

7.2	Transfer cells to cell culture hood	Take cells from the shaker and place them into the sterile Class II/laminar flow hood. Observe the Erlenmeyer flask for contamination before use. This can normally be identified by the discolouration of the media and visible clumps.
7.3	Count the cells. Dispense cells for counting into a bijoux and observe the phenotype under a microscope.	Take 1 ml of the cell culture using a serological pipette and place into a bijoux for cell counting. If the cells appear dense then further dilute the cells using culture media (e.g. 1 in 5). Do not forget to take this into account when calculating the cell density. Additionally, you can check your cells under a microscope to confirm no contamination is visible and that the cells are healthy. Take a sample from the bijoux and seed a 35mm cell culture dish. Healthy cells appear rounded, refractile, bright, clear and not clumped (see Figure 2).
7.4	Count the cells using a haemocytometer or cell counter.	For cell counting, take a sample of cells from the bijoux using a pipette and load into a prepared haemocytometer chamber using capillary action, attaching cover slip firmly to form the counting chamber. Count all the cells within the central 5 x 5 square grid on the counting chamber using a phase-contrast microscope (x 10 objective). Count cells touching the etched triple line on the top and left of the squares. Do not count cells touching the triple lines on the bottom or right of the squares. If the cells are clumped, they should be dispersed by gently pipetting up and down to get single cells before counting; otherwise the cell count will be inaccurate. Ideally you need at least 30 cells and no more than 100. We recommend taking the average of two independent readings. The cell count = number of cells x inverse of dilution factor x 10^4 /ml Alternatively, use a commercial cell counter and follow the manufacturer's instructions.
7.5	Determine the viability of the culture (optional step) using Trypan Blue staining and a phase-contrast microscope.	Cell viability should be tested from time to time during routine passaging and each time the cells are used for virus work (transfections or virus infection) to ensure good amplification of virus and/or high yield of proteins. This step can be performed in combination with 7.4 to generate a cell count and viability in one step. Prepare a 2% (w/v) preparation of Trypan Blue (e.g. Sigma-Aldrich®) in PBS. Dilute a sample of cells 1 in 2 with the stain (final concentration 1%) and immediately view cells using a haemocytometer (see above) under a phase contrast microscope. % viability = 100 - % dead cells % dead cells = total blue cells counted/total cells counted x 100 If also counting the number of cells, remember to take account of all the dilutions made.

		Or use a cell counter that automatically accounts for cell viability. **A good culture should be at least 95% viable.**
7.6	Seed the cells in ESF-AF medium to the required density for passaging the cells (see Table 4). [or to set up shake flasks for experimental work – see also Section 8] For routine sub-culture/passaging seed cells at **0.6 x 10^6 cells/ml**.	Based on the viable cell count, as determined in 7.3 – 7.5, calculate the volume of ESF-AF medium and the number/volume of cells needed to passage the SfC1B5 cells at **0.6 x 10^6 cells/ml** (see Table 4). A slightly higher density can be used if you need to expand cells more quickly (e.g. 0.8 x 10^6 cells/ml). For calculations use the following formula. **Cells** (Seeding density required [cells/ml] ÷ viable cell count [cells/ml]) X total volume = volume of cells required (ml) **Media** Total volume (ml) – volume of cells required (ml) = volume fresh medium required (ml) If reusing the same culture flask, remove the excess cells from the flask using a serological pipette, leaving the required number of cells (as calculated above). Then add the desired volume of pre-warmed ESF-AF medium to reach the correct density (Table 4). A flask can normally be re-used 2-3 times, however, if cells start clumping, use a fresh flask. If using a new flask, transfer the cells required (as calculated above) using a serological pipette and seed cells into a new flask. Add the desired volume of ESF-AF medium (as calculated above) to reach the correct density.
7.7	Incubate the shake flasks at 28°C on a rotating platform.	Incubate the newly seeded flasks on a rotating platform (as shake cultures) at an appropriate rpm to ensure good gas exchange (we use 120 rpm, 19 mm throw) in an incubator or warm room at 28°C. Ensure good aeration by not over filling the flasks. Approximately 20% (max. 25%) of total flask volume provides optimal surface area to volume ratio for gas exchange using vented lids (or non-vented lids kept loose).
7.8	Passage the cells 2-3 times per week.	The cell cultures need to be passaged 2-3 times per week; this is to prevent the cells from becoming overgrown (Figure 2, Table 4). Overgrown cells lead to increased clumping and cell death; also poor virus amplification or protein yields. If the cultures need to be left for more than 4 days (e.g. Thursday – Monday), the cells can be maintained at 26°C (or lower) to slow cell division (useful for long weekends and vacation times!).

Table 4. Seeding density for passaging/setting up suspension cultures

	SfC1B5 Cells	Notes
Seeding density for shake culture	0.6 – 0.8 x 10^6 cells/ml	Do not overfill flasks – have maximum surface area to volume ratio 20/25% of total volume
Passage shake culture when density reaches	4 - 6 x 10^6 cells/ml	Cells may reach higher density if required (see Figure 2), but repeatedly allowing the culture to reach stationary phase will have an adverse effect on cell performance over time and a new culture may need to be established

8. Experimental use of SfC1B5 cells for virus amplification and gene expression

After the SfC1B5 cells have been established by passaging a few times (Section 7), the cell culture can be expanded to be used for experimental work. Similar to Sf9 and Sf21 cells, SfC1B5 cells can be used for virus amplification and gene expression, with comparable results. We provide here some basic guidelines to help you in using SfC1B5, making a note of variations from Sf9 cells. However, if you require more information regarding specific protocols, including co-transfection, test expression, protein production, virus amplification, plaque assay and qPCR titration then these are available in OET's Handbook for using the *flash*BAC system, which is downloadable from the website (or for a PDF copy email info@oetltd.com). The Handbook is currently written for use of Sf9 cells – so the notes below on cell densities for SfC1B5 cells should be noted (we will be updating the Handbook to include SfC1B5 cells soon).

8.1 Guide to using SfC1B5 cells for virus amplification and gene expression

Step	Quick Steps	More detailed protocol and guidance notes
8.1	Count cells and check viability. (see Section 7)	It is important to note that cells for experimental use should be taken from mid-log phase cultures (4 -6 x 10^6 cells/ml) where the viability is >95%. Poor quality cells are a common reason for failure of virus to amplify to high titres or to ensure high yields of protein.
8.2	Seed the cells in ESF-AF medium to the required density for experimental work (Table 5)	Infection of SfC1B5 cells can be carried out using monolayer cultures or in shake cultures for protein production and virus amplification. Please note that SfC1B5 seeding densities are slightly lower than for Sf9 cells (Table 5). Based on the viable cell count, as determined in 8.1, calculate the volume of ESF-AF medium and cells needed to seed dishes/flasks at the required density for infection with recombinant baculovirus (Table 5). For seeding smaller volumes to be used for monolayer cultures (e.g. 35 mm dishes, 12/24 well plates), dilute the cells to the correct density in a sterile tube. Use a 10-25 ml serological pipette to carefully mix (not introducing bubbles) and dispense

		the cells into the monolayer dishes, using a 'figure of 8' to evenly disperse the cells. Incubate for at least 1 hour at 28°C to allow cells to adhere to the wells before infecting with virus. For shake cultures, directly add the media and required cells to a new flask of required size (from 125 ml flask to 5 L Fernbach) using a serological pipette (10 - 50 ml) to dispense the required number of cells. It is recommended to incubate the shake cultures for at least 1 hour (or overnight) at 28°C on a shaking platform. This allows for the cells to adapt to the fresh medium. If incubating the cells overnight, seed the cells at a slightly lower density, and check the cell count and viability before inoculating the cells with virus.
8.3	Inoculate the cells at the required multiplicity of infection (MOI) and incubate at 28°C.	All infections should be carried out under sterile conditions, preferentially in a Class II safety cabinet. Inoculate the cells at the required multiplicity of infection (MOI) for virus amplification or protein production. For virus amplification, the MOI will always be at 0.1 plaque forming units/cell (pfu or qpfu – qPCR e.g., using baculoQUANT™ from OET); this is to enable multiple rounds of virus amplification to derive high titres of virus (usually 1-3 x 10^8 (q)pfu/ml). For test expression or expression screening, different MOIs may be used to determine the optimal viral load for protein yield (we usually test 1, 3 and 5 or 10 pfu/cell). For protein production, use the optimum MOI but if MOI was not optimised, we would recommend using an MOI of 5 pfu/cell. Occasionally, lower MOIs give higher yields of some proteins and this has to be determined empirically. To calculate the required volume of virus, use the following formulae. **Virus required** volume of cells (ml) X cell density (cells/ml) X MOI = virus required for infection (pfu/cell) virus required for infection (pfu) ÷ virus inoculum (pfu/ml) = volume of virus inoculum required (ml) **An example:** To infect 1000 ml SfC1B5 cells at 1 x 10^6 cells/ml at MOI 5 (5 pfu/cell) requires 1000 x 1 x 10^6 x 5 pfu of virus = 5 x 10^9 pfu virus

		If the virus inoculum has a titre of 1 x 10^8 pfu/ml, you will require 50 ml virus inoculum to infect your culture. For shake cultures, the virus inoculum can be directly added to the cells, and then incubated for the desired time. For virus production this is normally 5 days, however cells should be monitored for signs of infection prior to harvest. Infected cells should look more, 'sausage' shaped and contain an enlarged nucleus. The cell viability will also drop, however, the SfC1B5 cells will usually show a higher viability after infection in comparison with Sf9 cells. For monolayer cultures, the cell culture medium is removed, and virus added (minimum of 100 µl). Incubate the cells for 1 hour using a rocking platform (to prevent drying out) and then replace the virus inoculum with fresh medium. Return cells to the incubator for the required time. For co-transfections, cells should be incubated for 5-7 days. Longer incubation times are sometimes required compared to when using Sf9 cells.
8.4	For virus amplification, harvest the culture medium containing virus and determine the virus titre using plaque-assay or qPCR. For protein production, harvest the culture medium (secreted proteins) or pellet the cells (intracellular proteins).	For shake cultures, transfer the cell culture medium into an appropriate centrifuge flask and pellet the cells at 4000 rpm, 15 minutes at 4°C using a floor-standing centrifuge. After centrifugation, decant the cell culture medium into a sterile container and label appropriately. Discard the cell pellet into waste for autoclaving. Remove a small sample of the virus to titrate by plaque-assay or qPCR. For gene expression/protein production, harvest the culture medium for secreted proteins and remove any cells as described above. For gene expression of intracellular proteins, pellet the cells and retain, decanting the medium into liquid waste and treating with Virkon® or autoclaving prior to disposal. For monolayer cultures, the cell culture medium (containing virus or secreted proteins) can be directly removed using a pipette and transferred to a microcentrifuge tube. Any cells can be removed by brief centrifugation and decanting the clarified medium to a fresh tube for storage at -20°C or for analysis. Infected cells can be harvested into the culture medium by pipetting up and down repeatedly. Infected cells tend to detach much more easily than non-infected cells. Transfer cells to microcentrifuge tube and pellet the cells, use a benchtop centrifuge at 4000 rpm, 5 minutes at 4°C. Remove the cell culture medium and process the pellet for analysis or storage at -20°C.
8.5	For short term storage of virus inoculum, store at 4°C or for long term	For short term storage then store the virus at 4°C in the dark. For longer term, store the virus at -80°C. It is beneficial to dispense the virus into smaller, single-use aliquots before freezing.

	storage, store at -80°C. Never store virus at -20°C.	The addition of FCS (to 2%) can be used to reduce the rate at which the virus titre drops over time and is advised for medium or long term storage at 4°C or -80°C. Where the use of FCS is not desirable, addition of sterile glycerol to 10% v/v works very well for long term storage at -80°C.

Table 5. Recommended seeding densities for virus amplification and gene expression

	SfC1B5 cells	Notes
Seeding density for co-transfection to make recombinant virus using a 35mm dish (12-well plate)	0.8 x 10^6 cells/dish (0.4 x 10^6 cells/well)	Seeding density is lower than advised for Sf9 cells in our *flashBAC* manual.
Seeding density for amplification of virus (P1, P2 etc) in shake cultures	0.5 - 1 x 10^6 cells/mL	Seeding density is lower than normally used for Sf9 cells for virus amplification
Seeding density for protein production in shake cultures	~1.3 x 10^6 cells/mL	Seeding density is lower than normally used for Sf9 cells for virus amplification
Seeding density for 35mm dish/6-well plate for test expression	1 x 10^6 cells/dish or well	Seeding density is similar to that advised for Sf9 cells in our manual.
Seeding density for 12-well plate for test expression	0.5 x 10^6 cells/well	Seeding density is similar to that advised for Sf9 cells in our manual.

For further details of protocols to prepare and analyse recombinant baculoviruses, please access our *flash*BAC manual downloadable from our website (www.oetltd.com) or request a spiral bound booklet format by contacting info@oetltd.com.