OXFORD EXPRESSION TECHNOLOGIES

Insect Cell Culture

A complete laboratory guide to insect cell culture

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Insect Cell Culture Manual 2024

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1. Introduction

1.1 Insect Cell Culture

This manual provides a guide to the growth and maintenance of insect cell cultures. It is extremely important that the insect cells used for the production and analysis of recombinant baculoviruses are of the highest quality. Insect cells can also be transfected with suitable plasmids to produce stable cell lines expressing a gene of choice. This manual provides tried and trusted protocols 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.

1.2 Choice of Cell Line

The insect cells most commonly used for the baculovirus expression system are *Sf*21 cells, originally derived from the pupal ovarian cells of *Spodoptera frugiperda* (Fall armyworm)[1]; *Sf9* cells, which are a clonal isolate of *Sf*21[2]; or *T. ni* (*T. ni* Hi5™) cells[3], originally derived from the ovarian cells of *Trichoplusia ni* (Cabbage looper)[4]. Generally, *Sf*21 or *Sf9* cells are used for co-transfections, virus amplification and plaque-assays. Whilst many labs use *Sf9* for all protocols, *Sf*21 cells are superior for plaque-assays and monitoring virus cytopathic effects and are more tolerant to sub-optimal conditions; and so ideal for those new to the system. *Sf9* cells are usually better for amplification of large stocks of virus and protein production and grow very well in large-scale fermenters or shake flasks. *T. ni* Hi5™ cells are often used to achieve maximal protein production but should not be used to produce or amplify virus because of the increased possibility of generating virus mutants[4]. For reasons that remain unclear, some genes are expressed much better in *Sf* cells than *T. ni* cells or *vice versa*, so testing expression in both cell lines at an early stage is recommended.

A number of engineered cell lines are available that may enhance expression of certain genes, particularly those that may be difficult to express in normal insect cells. For example, *super*Sf9 cells are genetically modified to express a stabilising protein and have increased longevity after virus infection, resulting in up to a 15-fold increase in protein yield compared to unmodified *Sf9* cells[5]. **Table 1** provides a summary of the main characteristic of each cell line.

NOTE

Superscripts 1-5 in Table 1 refer to references. *Foetal calf serum – batches vary; always test before using a new batch, some lots may require heat-inactivation at 60°C for 30 minutes.

1.3 Culture Medium

Most insect cell culture medium utilizes a phosphate buffering system, rather than the carbonate-based buffers commonly used for mammalian cells. This means that CO_2 incubators are not required. Serum is required for the maintenance of certain cell lines, but many have now been adapted to serum-free conditions. There is a large variety of insect cell culture media

available and it is beyond the scope of this manual to list them all; **Table 1** lists the media currently in use in our labs. *Sf*9, *Sf*21 and *T. ni* Hi5™ cells can all be grown in medium with serum or serum-free media. Always use a different bottle of cell culture medium for each cell line. The addition of antibiotics is optional (penicillin and streptomycin prepared with 5 units/mL^{-1} penicillin G sodium and 5 µL/mL^{-1} streptomycin sulphate in 0.85% saline can be used) but generally it is not recommended for virus amplification or protein production. Certainly it is best to maintain stock cultures without antibiotics; otherwise you may be maintaining a low-level contaminant that may later cause inefficient virus replication or protein production. Addition of antibiotics to plaque-assay medium is, however, recommended.

Table 1. Insect cell lines and characteristics

Cell Line	Appearance	Doubling Time	Uses	Media
*Sf*21[1]	Spherical, more irregular in size than *Sf*9 cells	24 hours	Plaque-assays (large, easy to see plaques form in 3-4 days) in serum-containing media	Serum-free (e.g. ESF 921™) Serum-containing such as TC100 with 10% v/v serum*
			Monolayer cultures	
			Suspension cultures	
			Making recombinant viruses	
			Amplification of virus	
			Production of proteins	
*Sf*9[2]	Spherical, more regular in size than *Sf*21 cells	24 hours	Suspension cultures	Serum-free (e.g. ESF 921™) TC100 plus 10% v/v serum*
			Monolayer cultures Making recombinant viruses	
			Amplification of virus	
			Production of proteins	
			Plaque-assays (small plaques form in 4 days)	

***super*Sf9 1-3**[5]	Spherical, more regular in size than *Sf*21 cells	24 hours +	Protein production for secreted or difficult to express proteins	Serum-free - adapted to *Sf*-900™ II (Gibco®) or; TC100 plus 10% v/v serum*
***T. ni* Hi5™**	Spherical, larger and more irregular in size than *Sf*9 cells	18 hours	Suspension cultures Production of proteins Monolayer cultures (loose attachment)	Serum-free (e.g. ESF 921™)

1.4 General Requirements for Insect Cell Culture

Insect cells have a relatively high dissolved oxygen content (DOC) requirement, particularly when infected with virus. 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. We recommend carrying out any cell culture work each day prior to handling virus and only using one cell line at a time.

1.5 Maintaining Cell Cultures

Insect cell lines can be maintained as either suspension cultures in shake flasks or stirred vessels (**Figure 1**), or in monolayer adherent cultures using vented/non-vented T-flasks or in dishes. Generally, insect cells adapted to serum-free medium are cultivated in suspension shake cultures whilst cells adapted to serum-supplemented media are cultivated in monolayer cultures or stirred suspension cultures (as growing these cells in shake culture generates excessive foaming and subsequent cell damage). However, cells grown in serum-free medium may also be cultured in monolayers.

Shake flasks may be recyclable glass or disposable. Stirred flasks are usually glass and contain either a magnetic stirring bar or suspended magnetic stirring rod (**Figure 1**). Both types are available from a range of suppliers. To maintain optimum cell culture conditions in a suspension culture, cell densities should be kept within certain ranges, i.e. within the log phase of growth (see **Table 2**, page **13**).

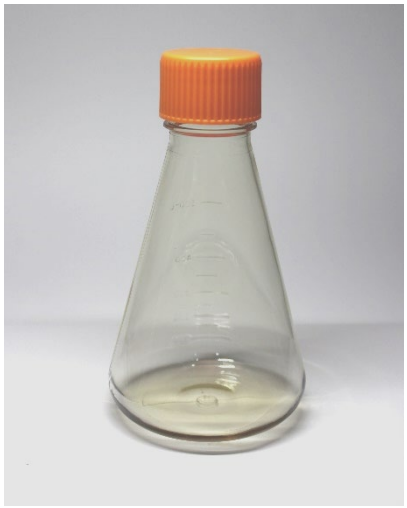

Figure 1. *Examples of insect cell culture flasks. (**A**) Suspension culture shake flask from 125mL to 3L (e.g. Corning™ Erlenmeyer flasks). (**B**) Suspension culture stirred vessel from 125mL to 1L (e.g. Techne biological stirrers).*

2. General Cell Culture Techniques

2.1 Sterile Technique

All techniques must be carried out using aseptic techniques and working in either in a class II safety cabinet or tissue culture laminar flow cabinet.

2.2 Passaging Cells

Sub-culturing, or passaging, of cells allows them to be maintained within log phase, preventing them from entering their stationary phase, and ensuring optimal viability for experimental use. Cells that are not in log phase will lack the enzymes and molecules needed for effective and efficient virus replication. The most likely problem with cells occurs when they have been allowed to reach stationary phase before passaging. At this point the cells have become 'stressed' and will begin to die, making recovery of passaged cells more difficult. Cultures that are continually left to reach stationary phase before passaging may suffer permanent problems and will not support virus replication. In this case, a new culture must be established from a frozen stock. Always check cells on a regular basis to prevent them from overgrowing.

Sub-culturing of shaker or stirrer cultures requires the seeding density of each cell culture to be determined beforehand. Generally, insect cell cultures can be passaged approximately 30 times before initiating a new culture from frozen stocks of cells; but this is a matter of preference for each lab. It is recommended to keep a record of the passage number on the culture flask.

When initiating a new culture from a frozen stock, we strongly recommend starting the culture as an adherent culture and once the cells are growing well (1-2 passages), transfer them into a

suspension culture. It is also easier to monitor cells visually under the microscope when growing in monolayer culture. It is possible to go straight from a frozen vial to a suspension culture but this requires a high density and high viability of the frozen cells. See notes later on in this manual.

2.3 Adherent Culture

Cells can be maintained in T25 or T75 flasks and grown at 27-28°C until the cells just reach confluency. Flasks can either be vented or non-vented, however if you are using the latter the lid of the flask must be kept loose to allow for proper aeration of the culture. Cells can be maintained in serum-free media or in media containing 10% serum (**Table 1**, page **6**). Confluency means the cells have just reached the point where they are touching each other, covering the entire surface of the culture vessel (**Figure 2**). Cells that are passaged repeatedly when the culture has past confluency will suffer from increased cell growth time and the appearance of grainy and 'sausage' shaped cells that have arrested mid cell division (**Figure 2**). Overgrown cells also have increasing numbers of cells floating in the medium; healthy cultures have very few floating cells. Attempting to passage cells before a culture reaches confluency means it is harder to dislodge cells as they are firmly attached to the flask surface and thus the chances of damaging the cells increase.

To sub-culture adherent cells, they should be detached from the surface by tapping the flask sharply on the bench top or palm of hand – this is the method we routinely use. Cells can also be detached by repeatedly pipetting the culture media over the monolayer to dislodge cells – use a Pasteur pipette.

NOTE

Cell scrapers can be used but in our experience this method leads to an excess of dead cells – home-made scrapers consisting of a bent glass rod with an attached piece of soft silicon tubing, and sterilised by autoclaving, are more gentle on cells than commercial scrapers.

Usually a 1 in 5 (*Sf*9) or 1 in 5-1 in 10 (*Sf*21/ *T. ni* Hi5™) dilution with 1 part old culture and 4 (or 9) parts fresh medium is sufficient to keep cells in a log phase culture. A typical 75cm^2 flask contains 10mL of culture medium and so 2mL would be transferred into 8mL of fresh medium for a 1 in 5 split (1mL plus 9mL for 1 in 10 split). These values are guides and will need to be adapted to suit the actual growth parameters in each lab.

It is also important not to make the density of cells too low when passaging, as cells need to be in reasonable proximity with other cells to promote growth. Some cells are more tolerant of this than others: *Sf*21 cells are the most tolerant, *Sf*9 cells are the least. **Figure 2** shows an example of cell density immediately after passaging and at confluent levels for *Sf*21, *Sf*9 and *T. ni* Hi5™ cells. If the cells in the newly seeded culture are too sparse, they may not divide and the culture will not become confluent.

It is essential to monitor cells under an inverted microscope prior to and after passaging to check for confluency, detachment after tapping, or any signs of poor health or over growth. Signs of poor health include: grainy cells, 'sausage' shaped cells, floating cells, longer doubling

time, failure to reach confluence. Grainy cells with refractive cuboidal-like structures in the nucleus are a sign of wild-type baculovirus contamination.

Maintain a log book of passaging and record the passage number, date and split ratio on the culture flask. After about 30 passages of being maintained in log phase, cultures start to lose viability and virus replication can be impaired. The old culture should be discarded and a new one established from a frozen stock. The log book can also be used to record any observations about the culture and this is sometimes very helpful when trouble shooting

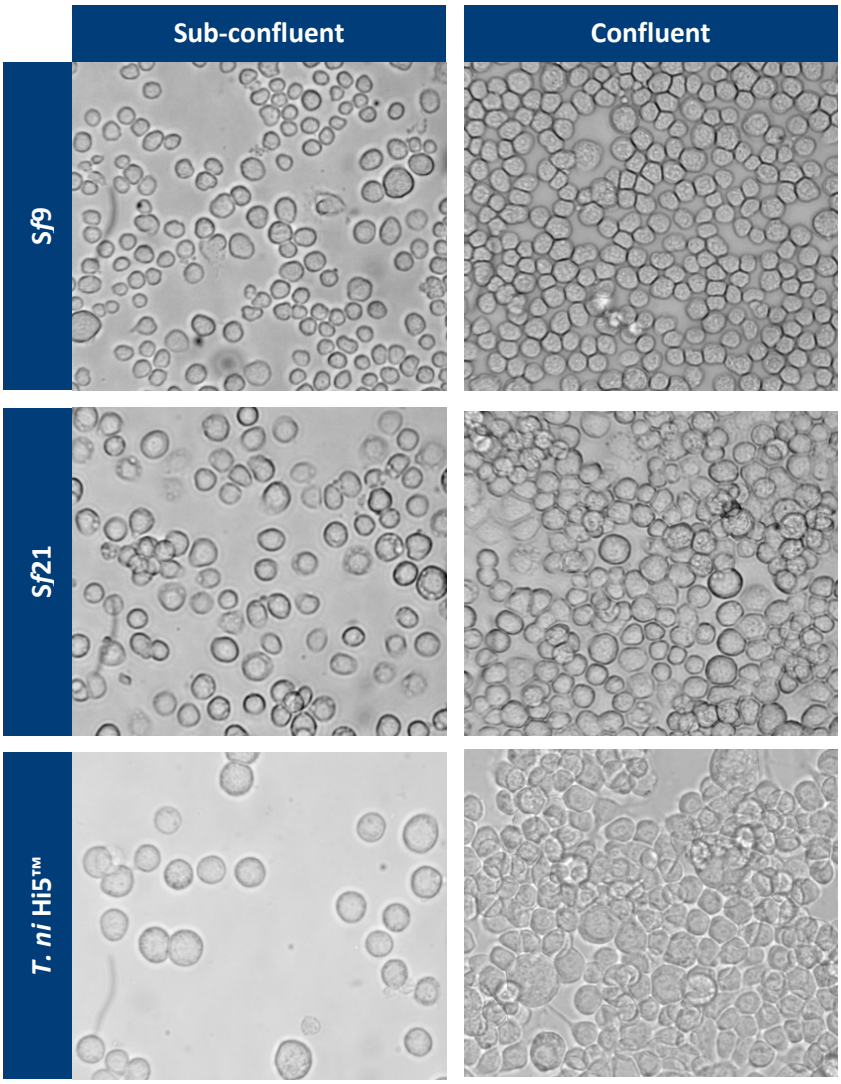

Figure 2. *Images to illustrate insect cells at various stages of culture. (**A**) Sf21 cells - sub-confluent (**B**) Sf21 cells - confluent (**C**) Sf9 cells - sub-confluent (**D**) Sf9 cells - confluent (**E**) T. ni cells - sub-confluent (**F**) T. ni cells – confluent.*

Whilst it is not essential, some labs prefer to count cells at each passage and seed a certain number of cells per flask each time. **Table 2** provides an indication of seeding density for passaging cells grown in monolayer culture.

When harvesting cells for use in experimental work, always count the number of cells and determine their viability so that the correct seeding density can be achieved for transfections or virus amplification.

Table 2. Seeding density for passaging monolayer cultures

Cell Line	Cells to Seed a T25 Flask	Cells to Seed a T75 Flask	Cells to Seed a T150 Flask	Split Ratio Guide (Culture : Fresh Medium)
***Sf*21**	1 x 10^6	3 x 10^6	5-6 x 10^6	1:5 to 1:10
***Sf*9/*super*Sf9**	1.5 x 10^6	5 x 10^6	1 x 10^7	1:5
***T. ni* Hi5™**	0.9 x 10^6	2-3 x 10^6	4-6 x 10^6	1:5 to 1:10
Culture Volume	5-7mL	10-15mL	30-40mL	-

Key points when culturing adherent cells:

- Check cells each day under the microscope until a confluent monolayer has formed
- Passage cells when confluent or shortly after
- Do not allow cells to become overgrown
- Do not split at too high a ratio
- Keep a record of passage number, date and split ratio
- Start a new culture from frozen stocks after about 30 passages
- Do not use antibiotics in routine cultures

2.4 Suspension Culture

Maintaining insect cells in suspension culture is very easy and provides a ready source of cells for amplifying recombinant viruses and infecting cells for protein production. There are two main methods for small-scale suspension cultures – shake flasks or stirred flasks (**Figure 1**, page **8**).

Shake flasks require the use of serum-free medium as otherwise serum creates excess froth that results in cells bursting as their membranes fuse with the bubbles. Most serum-free medium contain surfactants to reduce frothing. A surfactant such as Pluronic®F-68 can be added to media to reduce frothing but in our experience even adding surfactant to serum-containing medium does not prevent damage to cells.

There are commercial disposable shake flasks in a range of sizes that permit cultures from 10mL to 1.5L. As the culture volume increases attention must be given to aeration since insect cells, particularly those infected with virus, have a high oxygen requirement for metabolism. This can be achieved by selecting an appropriate rpm, not over filling the flask to maximise the surface area for gas exchange, and ensuring lids are vented or kept loose if using non-vented lids.

For those on a restricted budget, it is cheaper to use reusable glass flasks with cotton wool and loosely covered foil caps that have been sterilised by autoclaving and then dried. However, insect cells are very susceptible to contaminants in flasks and so any washing-up regime must be very stringent.

At OET our washing up regime is as follows:

- Disinfect flask with Virkon
- Soak in hot water with mild detergent (washing up liquid) and scrub internal surface with nylon bottle brush to remove adherent cell debris
- Rinse five times with hot water
- Soak in hot water for 2 hours
- Rinse in deionised water twice
- Soak in deionised water overnight
- Rinse in deionised water, dry and sterilise in an autoclave
- Dry in warm cabinet prior to use

As insect cells do not require CO_2, a shaking platform can be placed inside a standard incubator maintained at 27-28°C or even a clean cupboard/room maintained at this temperature.

Cells can also be grown in stirred cultures using commercial systems that have vessels with either a vertical impeller or hanging stirring bar that sit on a bespoke stirring device (**Figure 1**, page **8**). There are often side ports to take samples or add media/cells. Again, the caps should be vented or left loose and the vessels must not be overfilled to ensure good aeration. Cost effective home-made stirred flasks can be made using a flat-bottomed round flask with a stirring bar sat on a conventional magnetic stirrer. Again attention must be given to ensure the flasks are properly cleaned and sterilised before use.

Cells detached from a healthy adherent culture should be counted and used to set up a suspension culture according to the guidance in **Table 3**. It is important that the cells have a high viability – at least 95%. When establishing a suspension culture for the first time, set up a relatively modest scale flask (50-100mL culture). Once the suspension culture is established, a larger culture can be established if required.

Cells should be monitored daily by taking a small sample for counting and determining cell viability. In this way a growth curve can be plotted and monitored. Cells should be passaged before they reach stationary phase. An example of a typical growth curve is shown in **Figure 3**. Whilst we provide guidance on this matter, each lab should establish the optimal conditions for their cells and media combination.

Passaging cells in suspension is very easy. After counting and determining the viability, remove all the excess cells and, to the cells remaining in the flask, add fresh medium to establish a new culture at the correct cell density (**Table 3**). This can continue for up to about 30 passages (see notes under adherent cultures). After this time, the culture needs to be set up from a fresh adherent culture at low passage number.
The excess cells removed can be used to set up further suspension cultures for virus infection – either to amplify stocks of recombinant virus or infect cells for protein production. Cells can also be used to seed monolayer cultures for experimental use, e.g. 30mm dishes or multi-well

plates for plaque assay, co-transfections to make recombinant viruses, or to test expression levels.

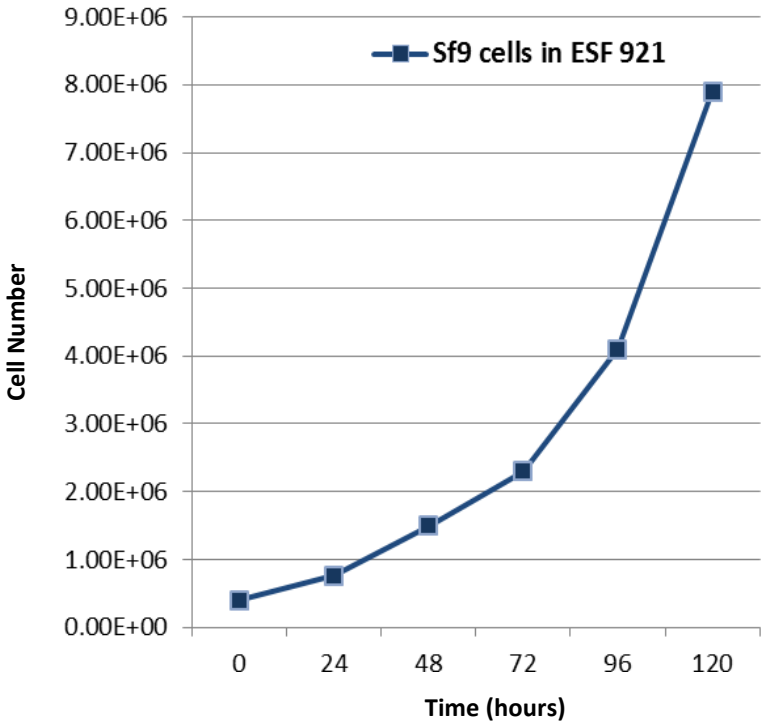

Figure 3. *Typical growth curve for Sf9 cells grown in suspension culture in ESF 921™ medium.*

Table 3. Seeding density for passaging suspension cultures

	*Sf*21 Cells	*Sf9* Cells	*T. ni* Hi5™ Cells	Notes
Seeding density for spinner culture (80-90rpm)	2-3 x 10^5 cells/Ml	Not usually grown in spinners	Not usually grown in spinners	Do not overfill flasks – maximum 50% of total volume
Passage spinner culture when density reaches	1-2 x 10^6 cells/ml	N/A	N/A	*Sf9* and *T. ni* cells are not usually grown in spinners
Seeding density for shake culture (135rpm)	4-5 x 10^5 cells/ml	4–5 x 10^5 cells/mL	2-3 x 10^5 cells/Ml	Do not overfill flasks – have maximum surface area to volume ratio 25% of total volume
Passage shake culture when density reaches	3-6 x 10^6 cells/mL*	3-6 x 10^6 cells/mL*	3-6 x 10^6 cells/mL*	*Cells may reach higher density if required

Key points when setting up a suspension culture:

- Use healthy log phase cells from an adherent culture that is at least 95% viable
- Count the cells and seed a culture no larger than 100mL using the guidelines in **Table 3** using serum-free medium
- Ensure good aeration by not over filling flasks, maintaining optimal surface area to volume ratio, using an appropriate rpm and vented lids/non-vented lids kept loose
- Monitor cells daily and set up a growth curve
- Passage cells by removing excess cells and adding fresh media to achieve correct cell density (**Table 3**) before cells reach stationary phase
- Use excess cells to start cultures for virus amplification, protein production, or experimental use in monolayer cultures
- Progress to larger volumes once cells established in culture
- Always note passage number, date and cell count/viability data on flask and in log book
- After 30 passages (from retrieval of frozen stock – include passage number data from adherent cultures), start a new adherent culture from a frozen stock
- Only use log phase cells for virus infections
- Do not use *T. ni* Hi5™ cells for making recombinant viruses or amplifying viruses

3. Counting Cells and Determining Cell Viability

3.1 Counting Cells

Before passaging cells or using cells for virus infection or transfections, cells should be counted to establish an accurate count per mL. This can be achieved using a commercial cell counter or by using a standard haemocytometer. When establishing a new culture, new cell line or using a new medium, it is well worth setting up a growth curve and monitoring cell density every 24 hours to establish the growth pattern.

The following provides a protocol for counting cells using a Neubauer haemocytometer.

Protocol:

1. Take a sample of cells from either a suspension or monolayer culture using a Pasteur pipette, load the prepared Neubauer chamber using capillary action, attaching cover slip firmly to form the counting chamber.

2. Count all the cells within the central 5 x 5 square grid (**Figure 4**) 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.

3. Count cells on both 5 x 5 grids and average the results. If the cells are too dense to count accurately, dilute the sample an appropriate amount to get a countable number of cells. If the cells are clumped, they should be dispersed by gently pipetting up and down to get single cells; otherwise the cell count will be inaccurate. Ideally you need at least 30 cells and no more than 100.

4. The 5 x 5 square gives the number of cells present in 0.1µL of culture. To calculate the number of cells per mL, multiply by 10,000 (10^4). If the cells were diluted before counting, remember to multiply the answer by the dilution factor.

Key points when counting cells:

- Ensure cells are in a single cell suspension and not clumped
- Take an average cell count across a minimum of 3 samples
- Don't forget to multiply answer by any dilution factor

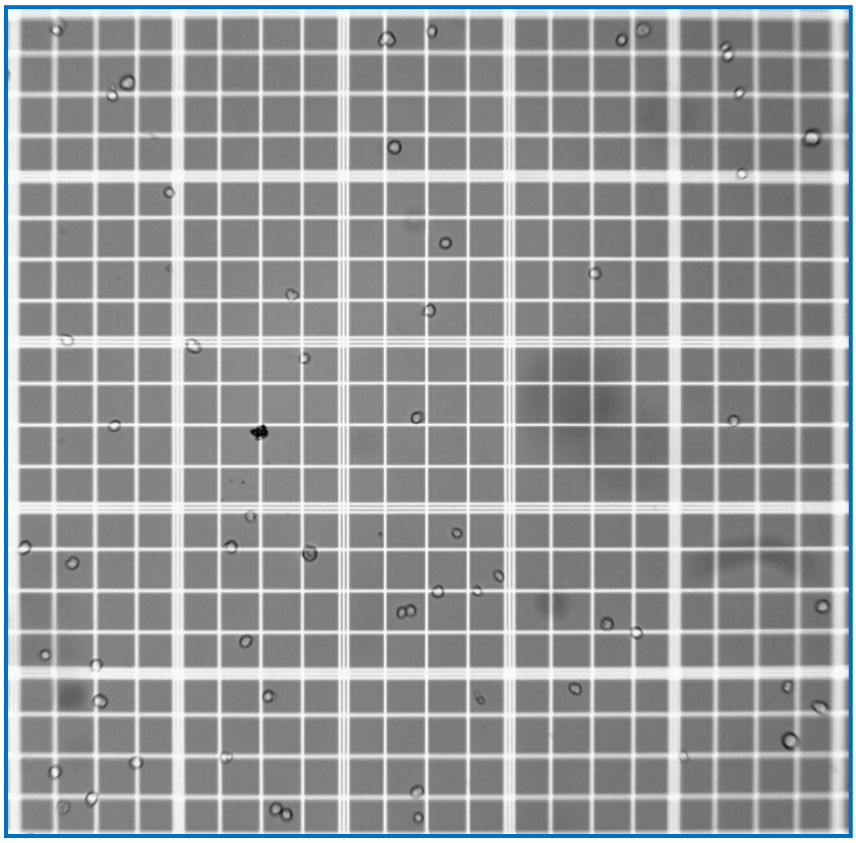

Figure 4. *Typical field of view when counting Sf9 cells using a Neubauer counting chamber.*

The number of cells counted in this field of view = 56 so the cell density for this sample would be 56 x 10^4 x 5 (as sample was diluted 1 in 5 to count) = 2.8 x 10^6 cell/mL. Note the sample was not stained with Trypan Blue so this count represents total cell numbers. It is recommended you take an average cell count across a minimum of 3 samples.

3.2 Cell Viability

Cell viability should be tested from time to time and every time cells are used for virus work (transfections or virus infection) as poor viability is a common reason for failure of virus to amplify to high titres or ensure high yield of protein production.

The easiest method is Trypan Blue exclusion and this can conveniently be performed when counting cells. Trypan Blue is a vital stain that is actively excluded from live cells; therefore, dead cells take up and retain the stain appearing blue under the microscope. Healthy cells appear re-fractile, bright and clear. The percentage of dead cells can be calculated and used to determine the overall viability of the culture. A good culture should be at least 90% and preferably 95% viable.

Protocol:

1. Prepare a 2% (w/v) preparation of Trypan Blue (e.g. Sigma-Aldrich®) in PBS.

2. Dilute a sample of cells 1 in 1 with the stain (final concentration 1%) and view cells under a phase contrast microscope immediately. It is convenient to count the cells at this stage (see above protocol).

3. % viability = 100 - % dead cells
   % dead cells = total blue cells counted/total cells counted x 100

Key points when determining viability:

- Ensure cells are in a single cell suspension and not clumped
- Count at least in duplicate to get an average
- Don't forget to multiply answer by two to take account of adding stain
- Don't leave cells in stain for more than few minutes before determining viability

4. Freezing and Thawing Cells

4.1 Freezing Cells

Once a cell line has been established in either adherent or suspension culture, after 2-3 passages, stocks should be frozen in ampoules in liquid nitrogen so that new cultures can 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 lower passage number as possible.

There are many variations in the protocols published for the freezing down and thawing of insect cells. The following is one that we find works well.

Method:

1. Set up a culture (suspension or adherent but suspension is better) that will provide sufficient cells for freezing down (1 x 10^7 cells per vial). Freeze down several vials in one batch – at least 20.

2. Harvest cells from a log phase culture (mid log phase for suspension or just prior to confluency for adherent cells). Count and determine viability. Cells need to be at least 95% viable.

3. Place and label the required number of cryovials on ice.

4. Pellet required number of cells very gently at 500rpm for 5 minutes. Remove and use the conditioned growth medium to prepare the freezing mixture as follows:

Serum-free medium*	**Serum-containing medium**
45% conditioned growth medium	40% conditioned medium
45% fresh medium	10% serum (e.g. foetal bovine serum)
10% DMSO	10% DMSO
	40% fresh medium

*use same freezing mix as serum-containing medium if preferred

5. Re-suspend the required number of cells in freezing mixture by very gently pipetting up and down to achieve a density of 1 x 10^7 cells/mL. Place 1mL aliquots into cryovials.

6. 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.

   Or, place the vials at -20°C for one hour and then at -80°C overnight before placing in liquid nitrogen.

7. After a few days, retrieve one vial to ensure that the freezing process has been successful.

8. Keep a log book/e-record of where and when each cell line is frozen, and when vials are recovered.

Key points when freezing cells:

- Use healthy log phase cells with 95% or greater viability
- Once DMSO has been added, cells must be cooled immediately to avoid damage
- Freeze slowly to avoid damage to cells
- Check process has been successful after a few days of storage
- Check liquid nitrogen levels regularly to ensure cells don't start to thaw during storage

4.2 Thawing Cells

On receipt of any frozen stocks of cells it is essential that they are either transferred to liquid nitrogen for storage or thawed to initiate a live cell culture. At OET we recommend initially starting the cells as an adherent culture and once they are growing sufficiently (1-2 passages), transfer them to a suspension culture. You must use aseptic techniques throughout and work in a class II safety cabinet or tissue culture laminar flow cabinet.

Protocol:

Adherent Culture

1. Defrost the cells rapidly in a clean 37°C water bath until just thawed. Decontaminate the outside of the vial by spraying with 70% ethanol before transferring to a sterile 30mL universal tube containing 10mL of culture medium. Perform a cell count and record the initial cell viability. DMSO is cytotoxic when cells are thawed so it is important to transfer them to fresh media as soon as possible.

2. Divide the 10mL between two T25 flasks to give a final volume of 5mL in each flask. Transfer the flasks to a 28°C static incubator and allow the cells to attach for 45-60 minutes. If using non-vented flasks keep the lids loose to allow for sufficient aeration of the culture.

3. Monitor the cells under the microscope. Live cells will have attached firmly while dead cells will float. Remove the freezing medium and replace with 5mL of fresh growth medium. If cells have not attached after 1 hour, return to incubate overnight.

4. Continue to incubate the cells until they have reached >80% confluency before sub-culturing. See page **9** for advice on the correct confluency for passaging insect cells.

5. Once a confluent monolayer has formed release cells from the flask's surface by tapping the flask sharply against the bench or palm of hand until >75% of the cells have detached. Cells can also be detached by repeatedly pipetting the culture media over the monolayer to dislodge cells. Do not use a cell scraper to dislodge cells. Transfer 2mL into a new T25 flask containing 3mL of fresh medium (this will create a second adherent culture) and sub-culture the remaining T25 flasks as described in the OET Insect Cell Culture User Guide.

Suspension Culture

1. To start a suspension culture, release the cells from two T25 monolayer cultures and transfer the entire volume from one flask, and 3mL from the second flask, to a 125mL shake flask. Count the cells and dilute with fresh growth media to give a final cell count of 1 x 10^6 cells/mL. The total volume in the flask should be between 15-30mL. Use the remaining 2mL in the second T25 flask to continue the cell line as an adherent culture.

2. Incubate both flasks at 28°C, leaving the 125mL flask on an orbital shaker platform rotating at 100-110 rpm. If using non-vented flasks keep the lids loose to allow for sufficient aeration of the culture.

3. Allow the cells to grow for 3-4 days. Count the cells from the suspension flask and transfer to a new 125mL flask the volume of cells necessary to reach a seeding density of 1 x 10^6 cells/mL in 25mL.

4. Once a suspension culture has been established and a cell density of 3-6 x 10^6 cells/mL has been reached *Sf*9 cells are routinely diluted to a density of 0.5 x 10^6 cell/mL. It is recommended that suspension cultures are passaged 2-3 times and are doubling

approximately every 24 hours with a viability of >90%, before used for experimental virus work.

5. We recommend that as soon as possible a batch of expanded cells are frozen down in liquid nitrogen to act as a source of low passage number cells for long term use.

Key points when thawing cells:

- Thaw quickly in a clean water bath
- Sterilise the outside of the vial before opening
- Set up a monolayer culture first so it is easy to remove dead cells
- When cells are growing well, establish suspension cultures

5. Adapting Cells to New Media

On occasion it is necessary to transfer cells into a new culture medium. Sometimes this can be achieved by simply using the new medium in place of the old. However, more often a period of adaption is required.

The accepted standard protocol is to culture the cells in 75% old medium: 25 % new for one to two passages, then 50% old: 50% new for 1-2 passages followed by 75% new: 25% old before finally culturing the cells in the new medium. This takes time but ensure the cells adapt well to a new medium.

A quicker version of this method can often be successful. It simply involves culturing the cells in 50% old medium: 50% fresh medium for one passage (monolayer or suspension culture) and then continuing to grow the cells in the new medium for at least one passage before using them for experimental work (e.g. amplifying virus or making recombinant viruses).

For this quicker protocol to be successful, it is important that the cells are in log phase of growth (80% confluent for monolayer or 3-5 x 10^6 cells/mL for suspension cultures) and that you monitor the cells each day to ensure they are still growing well.

The cells should be passaged as soon as they are confluent (monolayer cultures) or when they reach a density of 4-5 x 10^6 cells/mL (suspension culture). If the cells take a very long time to reach confluency or a density of 4-5 x 10^6 cells/mL, or cell viability drops very low, the longer adaption process described above will need to be followed.

5.1 Adapting Cells to ESF 921™

We have successfully adapted *Sf9* cells to ESF 921™ (from a variety of media) using the quick adaption protocol described below.

Protocol:

1. Take a log phase culture of the cells to be adapted (80% confluent monolayer culture) or cells at 3-5 x 10^6 cells/mL for suspension cultures. Ensure the culture is at least 90% viable.

2. For suspension cultures, dilute the culture with an equal amount of the new medium and continue growing the cells until they reach 4-5 x 10^6 cells/mL.

   For monolayer cultures, passage the cells using 50% old medium saved from the previous culture and 50% new medium. Grow until the cells are just confluent.

3. Passage the cells as normal using the new medium.

4. Use cells for experimental work after 1-2 passages in the new medium.

6. Mycoplasma

Mycoplasmas are bacteria that can often be present in cell cultures and affect their growth. As they lack a cell wall around their membrane they are resistant to many antibiotics such as penicillin and are very small and difficult to detect using conventional microscopes. While contamination with mycoplasma is less common in insect cells, mammalian cell cultures are particularly susceptible.

If you obtain any cell cultures from a source that cannot certify them as mycoplasma-free you should initially quarantine them until you can perform the appropriate test. There are a variety of methods to do this but in our experience one based on quantitative PCR works best. Cells should be tested a minimum of every 6 months to ensure they are clean of mycoplasma.

Should your cultures become contaminated it is possible to treat with specialised antibiotics, but our advice would be to discard the infected cells and obtain a new sample from a reputable source.

7. References

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All our baculovirus products have been optimised to ensure rapid and straightforward generation of recombinant virus, gene expression, and protein production. We offer everything you might need, from the revolutionary *flash*BAC™ baculovirus expression vectors and baculovirus qPCR titration kit, to transfection reagents and transfer plasmids. In addition we also stock a variety of *Spodotptera frugiperda* insect cell lines and culture media.

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