Protein Expression in the *Drosophila* Schneider 2 Cell System

Overexpression of recombinant proteins in cultured cells continues to be a convenient way to produce proteins in large quantities. The assorted limitations of bacterial, yeast, and mammalian protein overexpression systems have led to the exploitation of insect cell systems for the overexpression of functional proteins from a variety of sources. In contrast to the transient, baculoviral-aided overexpression of recombinant proteins in insect *Sf*9 cells (Bernard et al., 1999; Murphy and Piwnica-Worms, 1999), Schneider's embryonic *Drosophila* cell line 2 (S2 cells) can be used for stable expression of a variety of recombinant proteins using vectors specifically engineered for protein expression in *Drosophila* cells (see Tables 4.16.1 and 4.16.2). Thus, the S2-based system has all the advantages of the Sf9-based system with the major additional advantage that S2 cells can be used to attain stable expression under the control of strong, inducible promoters.

Basic Protocol 1 describes a procedure for efficient transfection of plasmid DNA into *Drosophila* S2 cells for the purpose of stable overexpression of target proteins. Basic Protocol 2 describes the activation of inducible promoters in S2 cells. Conditions for culturing and storing S2 cells are detailed in Support Protocol 1. Support Protocol 2 details the preparation of M3 medium, which is necessary for culturing *Drosophila* S2 cells.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

PREPARATION OF STABLE POLYCLONAL S2 CELL LINES

BASIC PROTOCOL 1

Plasmid DNA can be efficiently and reproducibly introduced into S2 cells by the calcium phosphate precipitation method of transformation (*APPENDIX 1C*). Although for S2 cells some researchers report that calcium phosphate systems based on *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer yield higher transformation efficiencies than their HEPES-based counterparts (Cherbas et al., 1994), the author's experience is that there is not a major difference. Because transformation efficiency is critically dependent upon the narrow pH optima of the calcium phosphate/DNA solution, commercially available, quality control–tested transfection kits are recommended in order to obtain reproducible results.

Materials

Schneider 2 (S2) at or near log phase cells (see step 3 or 15 of Support Protocol 1)
Complete M3 medium (see Support Protocol 2)
Plasmid DNA purified such that $OD_{260/280} \geq 1.8$
N,*N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer–based calcium phosphate transformation reagents (e.g., Stratagene)
70% (v/v) ethanol
Incomplete S2 medium (e.g., Life Technologies)
100× solution of selection drug (optional): e.g., 100 mg/ml geneticin (G418) or 20 to 30 mg/ml hygromycin B

150-cm^2 polystyrene tissue culture dishes
17 × 100–mm 14-ml tubes (Falcon #2059)
50-ml conical polypropylene centrifuge tubes with caps
Cell lifter
75-cm^2 screw-cap (nonaerating) polystyrene tissue culture flasks
27°C incubator

Contributed by John A. Schetz and Eswar P.N. Shankar
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Table 4.16.1 Plasmids Carrying Drug Resistance Genes for Use in Selection of S2 Cells

Plasmid name	Drug resistance gene	Drug resistance conferred	Vector backbone	Promoter	Poly(A)$^+$ signal	Reference
pHGCO	*E. coli* dihyrofolate reductase	Methotrexate	pBR322	*Drosophila* COPIA 5′ LTR	Early SV40	van der Straten et al. (1987)
pCodHygro	*E. coli* hygromycin B phosphotransferase	Hygromycin B	pBR322	*Drosophila* COPIA 5′ LTR	Early SV40	van der Straten et al. (1987)
pUChsneo	*E. coli* neomycin phosphotransferase	Geneticin (G418)	pUC8	*Drosophila* hsp70	None specified	Steller and Pirrotta (1985)
pCo*neo	*E. coli* neomycin phosphotransferase	Geneticin (G418)	pBR322	*Drosophila* COPIA 5′ LTR	Early SV40	van der Straten et al. (1987)

Additional reagents and equipment for culturing S2 cells (see Support Protocol 1) and testing expression (see Basic Protocol 2)

Transform S2 cells

1. Seed 20 ml S2 cells at a density of 1 to 2×10^6 cells/ml in complete M3 medium at room temperature into 150-cm^2 polystyrene tissue culture dishes.

2. In a 17 × 100–mm Falcon tube, mix sufficient purified plasmid DNA and freshly prepared BES-based calcium phosphate solution such that each of two to five dishes will receive 1 ml of solution containing 20 µg plasmid DNA. When cotransfecting with a drug resistance gene, use a 20:1 ratio of target gene plasmid to drug resistance gene plasmid.

   Increasing the ratio of target to drug resistance DNA significantly increases the number of copies of target DNA in the resulting drug-selected polyclonal population. Although the correlation is generally not 1:1, the greater the target copy number per cell, the more target protein expressed. Despite this, the number of copies for any given cell within a polyclonal population can vary up to 10-fold, and even cells with the same copy number can display variable levels of expression.

   Some researchers recommend decreasing the total amount of DNA by 4-fold in order to maximize transformation efficiencies in S2 cells.

   As in any calcium phosphate transformation, the physical size of the calcium phosphate/DNA crystal complex is a major factor determining transformation efficiency. If crystals are the appropriate size, the calcium phosphate/DNA solution should immediately appear slightly cloudy with a faint gray-blue tint (the Tyndall effect), and no large precipitates should be present at the bottom of the tube following the 20-min incubation recommended by the manufacturer. To avoid the formation of large precipitates, bubble air through the 2× BES buffer as the calcium phosphate/DNA solution is added dropwise.

3. Evenly add 1 ml calcium phosphate/DNA precipitate solution dropwise to each dish, swirl lightly to mix, and replace the dish lid.

   Increasing the volume ratio of medium to calcium phosphate/DNA solution will generally reduce transformation efficiency. Decreasing the volume ratio to <10:1 reduces viability due to calcium shock.

4. Sterilize 2 × 12–in. strips of Parafilm by wiping with 70% ethanol and allowing them to dry in a sterile hood. Seal the lids of the dishes by carefully but tightly stretching the Parafilm around the perimeter of the dish.

   The sealing step is necessary to prevent desiccation and to minimize the possibility of mold contamination. Alternatively, dishes can be stored in an airtight container (e.g., Tupperware).

Table 4.16.2 Protein Expression Vectors for Use in S2 Cells

Plasmid name	Vector backbone	Promoter	Poly(A)+ signal	Unique sites in MCS[a]	Other features	Reference
pRmHa-3	pUC18	*Drosophila* Mtn	*Drosophila* Adh	*Eco*R1, *Sac*1, *Kpn*1, *Sma*1, *Bam*H1, *Sal*1	None	Bunch et al. (1988)
pJACKS-3tag	pUC18	*Drosophila* Mtn	*Drosophila* Adh	*Bam*H1, *Apa*1, *Avr*2, *Stu*1, *Sal*1	*N-terminal features:* influenza virus hemagglutinin signal sequence; *Drosophila* Kozak's consensus sequence; FLAG tag; 6× polyhistidine tag; prothrombin protease cleavage site; S-protein tag; enterokinase protease cleavage site	Schetz et al. (2003)
pMT/V5-His A, B, and C	pBR322	*Drosophila* Mtn	Late SV40	*Kpn*1, *Spe*1, *Eco*R1, *Eco*R5, *Not*1, *Xho*1	*C-terminal features:* V5 antigen; 6× polyhistidine tag	SKB/Invitrogen
pDS47/V5-His A, B, and C	pBR322	DS47	Late SV40	*Kpn*1, *Sac*1, *Bam*H1, *Eco*R1, *Eco*R5, *Not*1, *Xho*1	*C-terminal features:* V5 antigen; 6× polyhistidine tag	SKB/Invitrogen
pMT/BiP/V5-His A, B, and C	pBR322	*Drosophila* Mtn	Late SV40	*Bgl*2, *Nco*1, *Sma*1, *Kpn*1, *Spe*1, *Eco*R1, *Eco*R5, *Not*1, *Xho*1	*N-terminal features:* *Drosophila* homolog of mammalian BiP signal sequence (HSC3, hsp72) *C-terminal features:* V5 antigen; 6× polyhistidine tag	SKB/Invitrogen

[a]MCS, multiple cloning site.

5. Store the dishes in the dark at room temperature for at least 24, but not more than 48 hr.
6. Transfer cells and medium to a 50-ml conical centrifuge tube. Add 25 to 30 ml incomplete S2 medium to the dish, gently dislodge any adherent cells with a cell lifter, and add this rinse to the 50-ml tube.
7. Centrifuge cells for 10 min at 225 × *g* at room temperature.
8. Decant and discard supernatant, and resuspend cells in 10 ml complete M3 medium supplemented with the appropriate concentration of selection drug from a 100× stock solution.

   Depending upon the drug-resistance gene co-transfected, 1 mg/ml G418 or 200 to 300 μg/ml hygromycin B may be used for drug selection.

   While high levels of protein expression can be reproducibly obtained from stable, polyclonal S2 cell lines selected with G418, which has been used as a selection agent in Drosophila for over a decade, there is concern by some researchers that a tiny fraction of untransfected S2 cells spontaneously accrue drug resistance. This unresolved issue may be related to the batch-to-batch variability of G418 preparations.
9. Add cell suspension to a 75-cm^2 screw-cap polystyrene tissue culture flask, cap the flask tightly, and incubate at 27°C.
10. Maintain cells under drug selection for 3 to 4 weeks before testing for expression of the target gene (see Basic Protocol 2). Once a week, add 5 ml complete M3 medium with the appropriate concentration of fresh selection drug. If cell densities are ≥5 × 10^6 cells/ml at the beginning of the third week, centrifuge cells and resuspend at 2 × 10^6 cells/ml in fresh drug selection medium.

Establish a stable polyclonal S2 cell population

11. Maintain selected polyclonal populations under constant drug selection for an additional 4 weeks, splitting cells 1:5 (v/v) about every 3 to 5 days. See Support Protocol 1 for storage and reculturing procedures.

The term stable refers to the stability of target gene expression levels over time for a given drug-resistant S2 cell population. Although drug-resistant S2 cells reportedly maintain their expression levels following removal of drug after the initial selection period, constant drug selection is recommended for long-term maintenance of stable polyclonal cell lines.

BASIC PROTOCOL 2

PROTEIN EXPRESSION BY INDUCTION OF EXOGENOUS TARGET GENES IN S2 CELL LINES

Although a variety of different promoters have been shown to be effective in *Drosophila* S2 cells, the *Drosophila* metallothionein (Mtn) promoter and the *Drosophila* heat shock protein 70 (hsp70) promoter are strong, inducible promoters. This protocol describes induction methods for the Mtn and hsp70 promoters. Both can be used in combination for cotransfected cells. If the *Drosophila* vectors being used are under the control of a constitutively active promoter (e.g., actin 5C distal promoter), this protocol can be omitted.

The hsp70 promoter has low levels of basal activity in S2 cells and can be activated by heat shock as well as other environmental stressors (e.g., >1 μM cadmium). In contrast, the basal activity of the Mtn promoter is virtually undetectable in S2 cells. The Mtn promoter is strongly induced by copper at concentrations that permit continuous S2 cell growth and protein synthesis (e.g., 500 μM). In contrast to copper, much lower concentrations of cadmium ions (e.g., 10 μM) are required to strongly induce the Mtn promoter; however, at these concentrations, cadmium ions also indirectly induce the hsp70 promoter as well as other *Drosophila* heat shock promoters.

NOTE: Because activation of heat shock promoters temporarily inhibits RNA splicing and the synthesis of non–heat shock proteins, induction by heat shock in cotransfected cells should be performed 1 to 2 days prior to induction of the target gene under the control of a non–heat shock promoter.

Materials

S2 cultures transfected with gene of interest (see Basic Protocol 1)
Complete M3 medium (see Support Protocol 2; for Mtn induction)
50 mM $CuSO_4$ *or* 1 mM $CdCl_2$ (for Mtn induction): ultrapure (e.g., Aldrich) and sterilized with a 0.22-μm filter
Aluminum foil (for hsp70 induction)
37°C incubator

Induce hsp70 promoter with heat shock

1. To reduce the risk of contamination, check that the lid of the S2 culture flask is tightly secured and completely seal the flask with fresh aluminum foil. Wrap the foil twice around the width of the flask, then crimp and roll the ends to seal.
2. Place the foil-covered flask in a 37°C bacterial incubator for 30 to 40 min.
3. Remove the flask from the incubator, discard the foil, and return the S2 cells to their previous culturing conditions.

While the hsp70 promoter has significant basal activity in S2 cells, strong induction requires heating shocking once about every 5 to 7 days.

Induce Mtn promoter with copper or cadmium ions

4. Split a near log-phase S2 culture 1:5 (v/v) in fresh complete M3 medium.

5. Add $^1/_{100}$th vol of 50 mM $CuSO_4$ (final 500 μM) or 1 mM $CdCl_2$ (final 10 μM).

   The optimal copper concentration for maximally inducing cells while maintaining high viability ranges from 0.3 to 1 mM. The variability is due to the batch-to-batch variability of the fetal bovine serum. Cells can also be maximally induced in the presence of 10 to 20 μM $CdCl_2$, but prolonged exposure to cadmium tends to be cytotoxic. At these concentrations, cadmium also activates heat shock promoters (e.g., hsp70) in Drosophila S2 cells.

6. Allow 8 to 72 hr for induction of protein expression before measuring protein expression levels by radioligand binding, immunofluorescence, or immunoblotting.

   S2 cells can be cultured continually in the presence of these concentrations of copper for at least 20 days.

CULTURE AND STORAGE OF *DROSOPHILA* S2 CELLS

SUPPORT PROTOCOL 1

Both short- and long-term strategies are available for culturing and storage of S2 cells. Although S2 cells grow rapidly at high densities in conditioned medium, it may take several weeks to reculture frozen stocks to a sufficient volume and density for rapid expansion.

Materials

Schneider 2 (S2) cells (ATCC #CRL-1963)
Complete M3 medium (see Support Protocol 2)
Cell culture–grade dimethyl sulfoxide (DMSO)
Incomplete S2 medium (e.g., Life Technologies)

25- and 150-cm^2 screw-cap (nonaerating) polystyrene tissue culture flasks
27°C incubator (or other dark, dry environment)
1.8-ml polypropylene cryotubes
Liquid nitrogen storage tank
15-ml conical polypropylene centrifuge tubes with caps
10-ml pipet

Additional reagents and equipment for counting cells with a hemacytometer (*APPENDIX 3B*)

Culture cells

1. Dilute near log-phase active S2 cultures ~1:5 (v/v) in complete M3 medium to yield cell densities ranging from ~1 to 3×10^6 cells/ml.

   Do not wash the cells prior to splitting; rather transfer the cells along with their conditioned medium. Ideally, S2 cell cultures should be seeded at cell densities ranging from ~0.5 to 3×10^6 viable cells/ml. Cells densities $<3 \times 10^5$ cells/ml result in drastically slowed doubling times or even complete stasis.

2. Add 25 ml diluted S2 cells to a 150-cm^2 screw-cap polystyrene tissue culture flask, tightly cap the flask to prevent aeration, and maintain at 27°C in a dark, dry environment.

   S2 cells can also be grown more slowly at room temperature (23°C), but lower temperatures also provide more favorable growth conditions for mold contamination. Prolonged culturing of S2 cells at temperatures >32°C is detrimental to cell growth, even though short exposures to higher temperatures are well tolerated (e.g., activation of heat shock proteins by heat shocking for 30 min at 37°C).

3. Continue to split actively growing cells 1:5 (v/v) about every 3 to 5 days.

   Under these conditions, ≥90% cell viability can be expected. When transferred to a fresh tissue culture flask, some S2 cells can be expected to adhere to the bottom of the flask, but most cells remain in suspension, especially at higher cell densities. In general, the longer S2 cells remain in culture in the same flask, the less cells will tend to stick to the flask.

Store over short term

4. Split log-phase S2 cell cultures (from step 3) 1:5 (v/v) in complete M3 medium.
5. Culture cells in a tightly capped flask in the dark at 18° to 25°C for up to 1 month.

   It is possible to culture S2 cells for longer than a month without changing the medium, as some cells remain viable through cannibalization of dying cells. However, infrequently changing the media tends to permanently decrease otherwise high expression levels.

Store over long term

6. Split log-phase S2 cell cultures (from step 3) 1:2 (v/v) in complete M3 medium and allow the cells to grow for an additional 24 hr at 27°C.
7. Use a hemacytometer (*APPENDIX 3B*) to check that the cell density is $\geq 1 \times 10^7$ cells/ml.

   It is important that S2 cells be frozen at high density, as they cannot be easily recultured at low density.

8. Suspend cells by swirling and transfer several milliliters of the newly log-phase culture to a 15-ml polypropylene tube.
9. Add ~10% (v/v) DMSO and then transfer 1.5-ml aliquots to 1.8-ml cryotubes.
10. Place cells in liquid nitrogen for long-term storage (up to 2 years).

Reculture frozen stocks

11. Quick-thaw 1.5 ml frozen S2 stock by incubating 3 to 5 min in a clean 37°C water bath.
12. Transfer cells to a 15-ml conical centrifuge tube and add 10 ml incomplete S2 medium.
13. Centrifuge for 10 min at $225 \times g$ at room temperature and decant the supernatant.

    This centrifugation step removes the DMSO.

14. Resuspend cells in 3 to 4 ml complete M3 medium by mild trituration with a 10-ml pipet, and then transfer to a 25-cm^2 tissue culture flask.

    Break the surface tension by lightly swirling so that the medium covers the entire bottom of the flask. Also make certain that the culture area is level.

15. Without splitting cells, continue to expand the cultures to ~30 ml by doubling the medium volume every 5 to 7 days with complete M3 medium.

    The resulting large-volume, high-density culture should be used as the cell splitting stock for large-scale expansion.

    For use in preparation of stable polyclonal cell lines (see Basic Protocol 1), cells must be diluted and passaged as described in steps 1 to 3. The number of passages is not critical, but the cell density and transfer of conditioned media is very important.

PREPARATION OF A MODIFIED SHIELDS AND SANG COMPLETE M3 MEDIUM

SUPPORT PROTOCOL 2

Drosophila S2 cells grow at high density and respond appropriately to heat shock when cultured in the Shields and Sang M3 medium supplemented with 5% to 20% (v/v) heat-inactivated fetal bovine serum (FBS). In 12% FBS-supplemented M3 medium (as described here), S2 cells grow primarily in suspension and can be expected to double about every 20 hr at 27°C, with a plateau in cell density at ~10 to 20 million cells/ml. Comparable results are achieved using a simplified, finely ground powder form of Shields and Sang M3 medium, which contains no Bacto Peptone and twice the amount of yeast extract as the original formulations (Shields and Sang, 1977).

While the suitability of serum for cell culture can vary considerably among different vendors or even from batch to batch from the same vendor, heat-inactivated serum that promotes robust growth of mammalian cell lines (e.g., CHO, C6 glioma, COS, HeLa, or Att20) will also promote robust S2 cell growth. Serum that has not been sufficiently heat inactivated or culturing under conditions favorable for mammalian cell culture (i.e., 37°C aerated with 5% CO_2) is cytotoxic to S2 cells. As some media components are temperature sensitive, it is important to store both dry and liquid media at 2° to 8°C.

Materials

Tissue culture–tested fetal bovine serum (FBS)
Powdered modified Shields and Sang M3 insect medium (e.g., Sigma)
Tissue culture–grade potassium bicarbonate ($KHCO_3$)
Penicillin/streptomycin at 5000 U/ml and 5 mg/ml, respectively

Clear resealable plastic bag (e.g., Ziploc)
65°C circulating water bath
50-ml polypropylene centrifuge tubes
250-ml, 0.45-µm tissue culture–grade sterile filtration devices with glass fiber prefilters
1-liter, 0.22-µm tissue culture–grade sterile filtration device with glass fiber prefilter

Prepare and sterilize heat-inactivated FBS

1. Seal a 500-ml bottle of room temperature, tissue culture–tested FBS in a clear resealable plastic bag.
2. Fill a circulating water bath such that the level of the water is a few centimeters above the serum level in the bottle, and preheat to 65°C.
3. Place the bottle of serum in the water bath and heat for 30 min.
4. Allow the serum to cool by placing it in a refrigerator for ~30 min.
5. Aliquot the cooled, heat-inactivated serum into 50-ml polypropylene centrifuge tubes and centrifuge at ≥1000 × *g* for 20 min at 4°C.

   This centrifugation step removes many of the serum proteins that precipitated upon heat inactivation, and primarily serves to aid in the subsequent sterile filtration step.

6. Place a glass fiber prefilter on top of a 250-ml, 0.45-µm sterile filtration device, apply a vacuum, and gradually pour 250 ml FBS supernatant onto the center of the prefilter. Repeat with a second filtration device and the other 250 ml supernatant.

   The prefilter prevents clogging. To maximize its effectiveness, make certain that the prefilter covers the entire surface area of the 0.45-µm filter, that a strong vacuum is applied before wetting, and that the wetting of the prefilter occurs from the center outward. Despite centrifugation and prefiltering, it will often still be necessary to use two sterile filtration units for every 500 ml heat-inactivated serum.

7. If heat-inactivated FBS is not to be used immediately, divide into 40-ml aliquots and freeze at −80°C for long-term storage (e.g., up to 2 years).

   Avoid freeze/thaw cycles.

Prepare and sterilize complete M3 medium

8. Carefully weigh out 39.3 g/liter powdered, modified Shields and Sang M3 insect medium.

   CAUTION: *Care should be taken not to inhale the finely powdered medium, which can be a powerful irritant and allergen.*

9. Dissolve in 900 ml distilled water by gently mixing in a 2-liter Erlenmeyer flask until the medium has completely clarified.

   Heat or hot water should not be used to dissolve the powdered medium, and prolonged exposure to light should be avoided, because the medium contains labile vitamins and amino acids. Highly concentrated stock solutions and media with a pH higher than ~7.2 cannot be made with these formulations due to the low solubility of some components.

10. Mix in 0.5 g $KHCO_3$.

11. Transfer medium to a 1-liter graduated cylinder and bring the total volume to 1000 ml.

12. Sterilize 875 ml medium, 125 ml heat-inactivated FBS, and 20 ml penicillin/streptomycin by filtration through a 1-liter, 0.22-µm sterile filtration device equipped with a glass fiber prefilter.

    Do not adjust pH, which should be between 6.5 and 6.9.

13. Store complete M3 medium up to 1 month in the dark at 2° to 8°C.

COMMENTARY

Background Information

A prerequisite for any detailed biochemical or biophysical study of protein structure and function is the availability of large quantities of homogeneous protein. The impetus for overexpressing proteins is simply to maximize the amount of starting material in order to minimize the subsequent protein purification task. For example, a 50-kDa protein expressing 50 pmol receptor/mg membrane protein would theoretically be pure after only a 400-fold purification.

The limitations on mammalian, yeast, and bacterial systems for protein overexpression has led to the exploitation of insect cell systems for the overexpression of functional mammalian proteins. The most notable insect-based overexpression system relies on the baculoviral-aided expression of recombinant proteins in *Sf*9 cell suspensions (Smith et al., 1983; Luckow and Summers, 1988). While high levels (typically 1 to 20 pmol/mg protein) of properly folded mammalian proteins can be expressed in *Sf*9 cells grown as high-density suspension cultures, the viral nature of the technique makes large-scale production laborious and inconvenient (i.e., by requiring the preparation of viral stocks for inoculation and accurate timing of the lytic cycle to maximize expression, while minimizing heterogeneity of the target protein). Both the initial advantages and the later-recognized disadvantages of the baculoviral Sf9 overexpression system prompted the development of a nonviral insect-based overexpression system. With increasing frequency, Schneider's embryonic Drosophila cell line 2 (S2 cell line) has been used to stably express a variety of recombinant mammalian proteins using plasmid vectors specifically engineered for protein expression in *Drosophila* cells. Thus, the S2-based system has all the advantages of the *Sf*9-based system with the major additional advantage that S2 cells can be used to make stable cell lines under the control of strong, inducible promoters.

S2 cells have a great protein-producing capacity, and for a variety of reasons they have proven to be remarkably effective hosts for the overexpression of recombinant proteins (see Table 4.16.3). First, S2 cells can be readily transfected with multiple copies of plasmid DNA that stably integrate into their chromo-

somes by P transposon–mediated integration, resulting in as many as 500 copies of integrated plasmid DNA per cell, depending upon the ratio of target plasmid to drug selection plasmid (Johansen et al., 1989). This high copy number accounts for the high overall levels of protein expression measured in polyclonal S2 populations. Second, S2 cells can be grown as high-density suspension cultures under conditions that require minimal maintenance and equipment. Third, protein expression can be tightly regulated with a *Drosophila* metallothionein promoter, which is strongly inducible and has virtually no basal activity in S2 cells. As a result of all of these factors, large amounts of homogenous recombinant protein can be produced with the S2 system in a relatively short time and with little equipment or space.

Historical perspective and origin of the S2 cell type

Until the early 1970s, *Drosophila* cell culture was limited to transient lines established from early-stage (6- to 8-hr) embryos. Using late-stage (20- to 22-hr) *Drosophila melanogaster* embryos, Schneider (1972) generated three genotypically distinct and genuinely stable cell lines. The second of Schneider's three cell lines (Schneider, 1972) is commonly referred to as S2 (in the older literature, S2 cells are sometimes referred to as Schneider's L2 cells or SL2 cells, with the L standing for line). Although Schneider originally described her cell line 1 (not S2) as having "macrophage-like" morphology, it is now also recognized that S2 cells assume macrophage-like morphologies in cultures using M3 medium (Kirkpatrick et al., 1995b). More convincing is that S2 cells undergo scavenger receptor-mediated endocytosis (phagocytosis; Abrahms et al., 1992), they produce antibacterial proteins (Samakovlis et al., 1990), and they grow better in M3 culture medium formulations containing more carbonate (Linquist et al., 1982), just like mammalian macrophages. A macrophage origin of the S2 cell type may also explain some of their unusual properties, such as cannibalization of dying cells and the preference for cells to grow in suspension even in unagitated and unaerated environments.

Overview of S2 cell promoters, polyadenylation signals, and signal sequences

A variety of different promoters, polyadenylation signals, and signal sequences have been tested in *Drosophila* S2 cells. The effectiveness of various promoters to induce the expression of recombinant genes follows the order: *Drosophila* hsp70 ≈ *Drosophila* Mtn ≈ *Drosophila* actin 5C distal > DS47 > COPIA ≈ α1-tubulin > Rous sarcoma virus (Di Nocera and Dawid, 1983; Steller and Pirrotta, 1985; Angelichio et al., 1991; Kirkpatrick et al., 1995b). The fibroin (Angelichio et al., 1991), early SV40 (van der Straten et al., 1987), and herpes thymidine kinase promoters (Steller and Pirrotta, 1985) are all essentially inactive in S2 cells. Both the hsp70 promoter and the Mtn promoters are strongly inducible, but only the hsp70 promoter has considerable basal activity in S2 cells (Di Nocera and Dawid, 1983; Rubin et al., 1993; Huebel et al., 1995)—i.e., enough to confer drug resistance in the absence of heat shock when controlling *neo* gene expression. Since conditions that strongly activate the hsp70 promoter also activate other heat shock promoters (e.g., heat shock or >1 μM cadmium; Bunch et al., 1988), it is not surprising that S2 cell expression vectors invariably use Mtn promoters to control target gene overexpression by copper induction.

Several polyadenylation signals are effective in S2 cells. The order of mRNA stability conferred by various polyadenylation signals is: late SV40 > *Drosophila* Mtn ≈ early SV40 ≈ *Drosophila* alcohol dehydrogenase (Adh; Bunch et al., 1988; Angelichio et al., 1991). Although the late SV40 polyadenylation signal is ~3-fold more efficient in S2 cells, all of these polyadenylation signals result in significant levels of target gene protein expression. Recently, the N terminus of human tissue plasminogen activator (tPA; Culp et al., 1991), the *Drosophila* homolog of the mammalian immunoglobulin heavy chain chaperone binding protein (BiP) (named HSC72; Kirkpatrick et al., 1995a), and the influenza virus hemagglutinin signal sequence (Schetz et al., 2003) have been shown to promote proper protein folding, trafficking, and/or secretion in S2 cells, making them efficient signal sequences.

Critical Parameters and Troubleshooting

S2 cell culture

Robust, stable S2 growth is most critically dependent upon cell density, temperature, light, and aeration. Maintaining S2 cells at high density (>0.3×10^6 cells/ml) is an especially critical parameter, as low-density cultures grow very slowly or arrest completely even when the other conditions are optimal. If it is not possible or

Table 4.16.3 Some Endogenous and Exogenous Proteins Expressed in S2 Cells

Protein class	Protein name	Source	Reference	Comments
GPCR[a]	M_1 muscarinic acetylcholine receptor	*Drosophila*	Millar et al. (1995) Yagodin et al. (1999)	Robust receptor expression; ~2.5 pmol/mg Imaging of intracellular Ca^{2+}
GPCR	M_2 muscarinic receptor	Pig	Graziano et al. (1998)	Robust receptor expression; ~5 pmol/mg
GPCR	$D2_L$ dopamine receptor	Rat	Schetz et al. (2003)	Robust receptor expression; ~10 pmol/mg. Stereoselective drug binding is similar to that in mammalian cells.
GPCR	D_1 dopamine receptor	Rat	Schetz et al. (2003)	Robust receptor expression; ~8.5 pmol/mg. Stereoselective drug binding is similar to that in mammalian cells.
GPCR	Glucagon receptor	Human	Graziano et al. (1998)	Ultra-high expression; ~100 pmol/mg. Anisotropy of fluorescent ligands.
GPCR	Tachykinin receptor (STKR)	*Stomoxy calcitrans*	Torfs et al. (2000)	Positively coupled to phospholipase C and adenylyl cyclase
			Torfs et al. (2001)	Receptor expression; ~0.15 pmol/million cells. Imaging of intracellular Ca^{2+}
GPCR	Octopamine receptor (OMAB)	*Drosophila*	Van Poyer et al. (2001)	Positively coupled to adenylyl cyclase and activated by the phentolamines: tyramine and octopamine
GPCR	Leucokinin receptor	*Drosophila*	Radford et al. (2002)	Immunocytochemistry and western blotting
GPCR	Inositol (1,4,5)-triphosphate receptor (IP_3R)	*Drosophila*	Pollock et al. (2003)	Ultra-high protein expression; ~400 mg/liter
GPCR	Interleukin 5 (IL-5) receptor	Human	Johanson et al. (1995)	Robust receptor expression; 17 mg/liter. The peptide ligand, IL-5, is also expressed at ~22 mg/liter.
GPCR	Chemokine(CXCR3)[b] receptor	Human	Hensbergen et al. (2001)	Expression of CXCR3 receptors is similar to that in mammalian cells
GPCR	Chemokine(CXCL11)[c] receptor	Human/ murine	Hensbergen et al. (2001)	Expression of CXCL11 receptors is similar to that in mammalian cells
Ion channel	γ-Aminobutyric acid receptor ($GABA_c$)	*Drosophila*	Millar et al. (1994)	Robust expression; ~3 pmol/mg Whole cell patch clamp
Ion channel	γ-Aminobutyric acid receptor (GABA)	*Drosophila*	Grolleau et al. (2000)	Single-channel recordings.
Ion channel	Nicotinic acetylcholine receptor	*Drosophila*	Lansdell et al. (1997)	Robust receptor expression; ~6.5 pmol/mg
Ion channel	Chloride channel	*Drosophila*	Asmild at al. (2000)	Endogeneous channels. Whole cell patch clamp recording gives an estimate of 0.02 channels/μM^2.
Ion channel	Voltage-dependent sodium channel	*Drosophila*	Meadows et al. (2002)	Patch clamp recordings
Ion channel	Transient receptor potential–like (TRPL)	*Drosophila*	Goel et al. (2001)	Immunoprecipitation and immunoblotting. Imaging of intracellular Ca^{2+}. Comparison of expression with *Sf*9 cells.
Enzyme	Chloramphenicol 3-*O*-acetyltransferase	Bacterial	Di Nocera and Dawid (1983)	Effective reporter protein
Enzyme	Galactokinase	Bacterial	Johansen et al. (1989)	Robust expression; ~3 mg/liter. Effective reporter protein.
Enzyme	Dihydrofolate reductase	Bacterial	Van der Straten et al. (1987)	Confers resistance to methotrexate
Enzyme	Hygromycin B phosphotransferase	Bacterial	Van der Straten et al. (1987)	Confers resistance to hygromycin B
Enzyme	Neomycin	Bacterial	Van der Straten et al. (1987)	Confers resistance to G418
Enzyme	Glucosidase II	*Drosophila*	Ziak et al. (2001)	Endogenous enzyme and only one isoform expressed

continued

Table 4.16.3 Some Endogenous and Exogenous Proteins Expressed in S2 Cells, *continued*

Protein class	Protein name	Source	Reference	Comments
Peptide	Neurotensin	Human	Friry et al. (2002)	Levels of mRNA transcribed similar to that in mammalian cells
Antibody	IgG_1 monoclonal antibody	Human	Kirkpatrick et al. (1995a)	BiP-mediated assembly of both heavy and light chains
Oncogene protein	H-ras	Human	Johansen et al. (1989)	H-ras is growth-inhibitory, thus an inducible promoter is required
Cytoskeletal protein	Cadherin	*Drosophila*	Dubreuil et al. (1999)	Western blotting
Cytoskeletal protein	Neuroglian	*Drosophila*	Dubreuil et al. (1999)	Western blotting
Glycoprotein	HIV–gp120[d]	AIDS virus	Culp et al. (1991)	Robust protein expression; ~35 mg/liter. Glycosylation is limited to simple core sugars.
Glycoprotein	Erythropoeitin (EPO)	*Drosophila*	Lee et al. (2000)	Robust protein expression is; ~12mg/liter
Apoptotic protein	Presenilin	*Drosophila*	Ye and Fortini (1999)	Western blotting
Apoptotic protein	Inhibitor of apoptosis (IAP1)	*Drosophila*	Muro et al. (2002)	Comparison of expression with that in *Sf*9 cells

[a]GPCR: G protein–coupled receptor.

[b]The alternative nomenclature for the chemokine (CXCR3) receptor is interferon inducible protein-10 (IP-10).

[c]The alternative nomenclature for the chemokine (CXCL11) receptor is interferon inducible protein-9 (IP-9).

[d]HIV: Human immunodeficiency virus.

practical to sufficiently increase the S2 cell density by reducing the volume of medium, lower-density cultures can sometimes be coaxed to divide by increasing the total cell density to 2 to 5×10^6 cells/ml with irradiated "feeder cells" (24-krad γ-irradiated cells at a density of 3×10^7 cells/ml; Ashburner, 1989; Cherbas et al., 1994). The irradiated feeder cells can no longer divide, but they condition the medium and serve as food when they die. As the M3 medium contains vitamins and amino acids that are sensitive to light, all forms of medium, and even cells contained in this medium, should be protected from prolonged exposure to intense light and stored in the dark. As some of these same medium components are temperature sensitive, it is important to store both dry and liquid media at 2° to 8°C. Serum that has not been sufficiently heat inactivated or cultured under conditions favorable for mammalian cell culture (i.e., 37°C aerated with 5% CO_2) is cytotoxic to S2 cells. The authors' experience has been that maintaining long-term cultures with minimal media changes will permanently decrease expression levels of the exogenous protein.

Transformation of plasmid DNA into S2 cells

While the BES-based calcium phosphate precipitation systems are recommended for S2 cell transformations since they are reported to be approximately ten times more efficient than the corresponding HEPES-based systems (Cherbas et al., 1994), the authors have not observed such differences in transformation efficiencies when using commercially available, quality-controlled HEPES-based kits. The use of 20 µg plasmid DNA per ml of calcium phosphate transfection reaction is prevalent, although some researchers contend that values between 2 and 6 µg of plasmid DNA per ml gives the highest transformation efficiencies (Ashburner, 1989).

Anticipated Results

A hallmark for a healthy population of actively dividing S2 cells is the presence of grape-like bunches of cells that appear as tumbleweeds under the microscope when the flask is agitated. This phenomenon is usually, but not always, observed at higher cell densities (e.g., $2\text{-}3 \times 10^6$ cells/ml). It is plausible, although unproven, that these bunches of adherent cells are a clonal population.

In general, increasing the ratio of target gene DNA to drug resistance gene DNA produces cell populations with a greater number of copies of target gene, and the greater the number of copies of the target gene, the higher the mRNA and protein expression levels for that gene. However, the relationship between number of gene copies, mRNA levels, and protein expression levels are by no means proportional. Interestingly, these relationships seem to rely most heavily upon the type of promoter driving target gene expression (van der Straten et al., 1987). Despite these variations, high levels of expression are achieved in stable, polyclonal populations (Table 4.16.3), even in cases where the target gene product is cytotoxic to the S2 cells (Culp et al., 1991).

Asparagine-linked glycosylation of recombinant proteins in S2 cells is primarily limited to simple core mannose structures containing glucose, *N*-acetylglucosamine, and occasionally fucose, with $Man_3GlcNAc_2$ being the most complex oligosaccharide (Hseih and Robbins, 1984; Culp et al., 1991). The reason for this is that insects apparently lack the necessary N-linked oligosaccharide processing enzymes for converting the core high mannoses to considerably more complex glycan structures, and they do not synthesize galactose or sialic acid. Remarkably the $Man_3GlcNAc_2$ oligosaccharide motif occurs on the same sites in proteins that correspond to the complex oligosaccharide glycosylation sites in mammalian cells (Hseih and Robbins, 1984). Apparently the peptide consensus sequences recognized by the most complex oligosaccharide processing enzymes in both insects and mammals are evolutionarily conserved.

Even if the S2 cells happen to express the *Drosophila* homolog of the mammalian target gene being expressed, it may still be possible to screen for recombinant receptor expression levels via high-affinity radioligand binding. The reason for this is that some *Drosophila* homologs (e.g., G protein–coupled receptors) are resistant or insensitive to certain pharmacological agents that bind their mammalian counterparts with high affinity and, in these cases, the binding of such mammalian radioligands in a *Drosophila* background would be very low. In general, mammalian proteins can be expected to display similar inherent properties when they are expressed in S2 cells and mammalian cell systems. However, no assumptions should be made concerning the promiscuity of *Drosophila* accessory proteins with recombinant mammalian proteins. For example, the pharmacological profile of mammalian D1 and D2 dopamine receptors is the same when they are expressed in S2 and mammalian cells, yet these mammalian GPCRs do not couple with *Drosophila* G proteins (Schetz et al., 2003).

Time Considerations

Because S2 cell growth is optimal in high-density cell suspensions, the production and use of stable, polyclonal populations of S2 cells for the overexpression of recombinant proteins is prevalent. It is possible to clone S2 cells by drug selection on agar plates supplemented with feeder cells, but this process is laborious and time consuming (~2 months additional time for colony formation and large-scale expansion of individual clones; Ashburner, 1989). Furthermore, the additional effort for cloning does not seem worthwhile, because the levels of expression reported for high-expressing clones (Millar et al., 1994, 1995) appear to be similar to those expected from a polyclonal population (Table 4.16.3).

On the one hand, it takes a considerable amount of time to establish a stable polyclonal population as compared to other overexpression methods (e.g., transient expression in COS cells or baculovirus infection of *Sf*9 cells). However, once a stable population is selected, large quantities of homogeneous target gene can be reproducibly expressed without the need to continuously transform or infect cells with the target gene. Finally, the target gene can be maximally induced by relatively brief exposures to inducing agents (e.g., 8 to 72 hr), and these maximal protein expression levels will persist for weeks in the continued presence of inducing agent. Consequently, only after a stable S2 cell line has been established will the S2 cell system afford a significant time and cost advantage over conventional overexpression systems. The time advantage of the S2 cell system increases with the number of experiments performed.

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Key References

Angelichio et al., 1991. See above.

The single most extensive comparison of the effectiveness of promoters and polyadenylation signals in S2 cells published to date. A must read for those attempting to design S2 cell expression vectors.

Ashburner, 1989. See above.

A comprehensive treatise on Drosophila laboratory techniques, including specific Drosophila cell culture techniques such as cloning procedures for S2 cells. Contains many detailed methodological tips referenced as personal communications with prominent Drosophila researchers.

Ashburner, M. 1989. *Drosophila*: A Laboratory Handbook. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Companion handbook for the manual listed above. In addition to being a good general resource on Drosophila, this book provides historical and factual details about vectors designed for use in Drosophila cells.

Drosophila Information Services (DIS).

A series of documents produced yearly from materials contributed by Drosophila researchers. Specific arrangements and bibliographies for this documentation service, as well as the institutions at which these publications are produced, vary according to volume. In most libraries, the DIS is listed as a journal by volume and year. The DIS is a compilation of short blurbs concerning recent methodological advances in Drosophila research, especially those related to Drosophila tissue culture.

Internet Resources

http://flybase.bio.indiana.edu

An extensive database resource for all that is Drosophila, including sequence information for various Drosophila vectors (transposons).

http://www.invitrogen.com/expressions/index.htmlis

Invitrogen's Web site, containing schematics of their Drosophila expression vectors.

Contributed by John A. Schetz
and Eswar P.N. Shankar
University of North Texas
Health Science Center
Fort Worth, Texas