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Drosophila Expression System

USER GUIDE

For the stable expression and purification of heterologous proteins in Schneider 2 cells

Catalog Numbers K4120-01, K4130-01, K4140-01, K5120-01, and K5130-01

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Contents

Kit contents and storage

Types of kits

This manual is supplied with the kits listed below.

Product	Catalog no.
DES™ Inducible Kit	
with pCoHygro	K4120-01
with pCoBlast	K5120-01
DES™ Inducible/Secreted Kit	
with pCoHygro	K4130-01
with pCoBlast	K5130-01
DES™ BioEase™ Expression Kit *with pCoBlast*	K4140-01

Shipping/Storage

The DES™ Reagents are shipped as described below. Upon receipt, store each component as detailed below.

Item	Shipping	Storage
Expression vector(s) and control vector	Room temperature	–30°C to –10°C **Note:** See vector manual for details
Drosophila S2 cells	Dry ice	Liquid nitrogen
Schneider's *Drosophila* Medium	Room temperature	2°C to 8°C
Selection vector (pCoHygro or pCoBlast)	Dry ice	–30°C to –10°C
Primers	Dry ice	–30°C to –10°C
Calcium Phosphate Transfection Kit	Dry ice	–30°C to –10°C
Copper sulfate (if supplied)	Dry ice	2°C to 8°C
Blasticidin (DES™ kits with pCoBlast only)	Room temperature	–30°C to –10°C
Hygromycin (DES™ kits with pCoHygro only)	Dry ice	2°C to 8°C, protected from light

Product use

For Research Use Only. Not for use in diagnostic procedures.

Continued on next page

Kit contents and storage, continued

DES™ expression vectors

Each DES™ kit contains an expression vector for cloning your gene of interest and a corresponding expression control vector. All kits except the DES™ BioEase™ Expression Kit also contain primers to facilitate sequencing. The table below lists the expression vector, control vector, and primers (if applicable) supplied with each kit.

Refer to the specific manual for each expression vector for detailed information about the amount provided and instructions on how to use the vectors. A manual specific for the expression vector is supplied with each DES™ kit, but may also be downloaded from **www.thermofisher.com** or by contacting Technical Support (see page 35).

DES™ kit	Catalog no.	Expression vector	Control vector	Primers
Inducible	K4120-01 K5120-01	pMT/V5-His A, B, and C	pMT/V5-His/*lac*Z	MT Forward BGH Reverse
Inducible/Secreted	K4130-01 K5130-01	pMT/BiP/V5-His A, B, and C	pMT/BiP/V5-His/GFP	MT Forward BGH Reverse
BioEase™ Expression	K4140-01	pMT/BioEase™-DEST	pMT/BioEase™-GW/*lacZ*	Not included

Primer sequences

The sequence of each primer is provided below:

Primer	Sequence	pMoles supplied
MT Forward	5´-CATCTCAGTGCAACTAAA-3´	368
BGH Reverse	5´-TAGAAGGCACAGTCGAGG-3´	358

pCoHygro selection reagents

All DES™ kits with pCoHygro contain the following selection reagents, **except as noted**. Store as indicated below.

Item	Concentration	Amount supplied	Storage
pCoHygro vector	0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	40 µL	–30°C to –10°C
Copper Sulfate*	100 mM	1.5 mL (sufficient for 100 inductions)	2°C to 8°C
Hygromycin-B	100 mg/mL	0.5 mL (50 mg)	2°C to 8°C, protected from light

*Copper Sulfate is used to induce the metallothionein promoter. It is not included in the DES™ Constitutive Kits.

Continued on next page

Kit contents and storage, continued

pCoBlast selection reagents

All DES™ kits with pCoBlast contain the following selection reagents, **except as noted**. Store as indicated below.

Item	Concentration	Amount supplied	Storage
pCoBlast vector,	0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	40 µL	–30°C to –10°C
Copper Sulfate*	100 mM	1.5 mL (sufficient for 100 inductions)	2°C to 8°C
Blasticidin	–	50 mg	–30°C to –10°C

*Copper Sulfate is used to induce the metallothionein promoter. It is not included in the DES™ Constitutive Kits.

Cells and medium

All DES™ kits include the following cells and medium:

- Schneider (S2) Cells: 1 vial containing 1 mL of cells at 1×10^7 cells/mL in Freezing Medium. **Store in liquid nitrogen upon receipt.**
- Schneider's *Drosophila* Medium for culturing S2 cells: 2 × 500 mL. **Store at 4°C upon receipt.**

Calcium Phosphate Transfection Kit

The Calcium Phosphate Transfection Kit included with each DES™ Kit contains the following reagents. Sufficient reagents are provided for 75 transfections. **Store at –20°C.**

Note: The Calcium Phosphate Transfection Kit is also used to transfect mammalian cells. The positive control vector pcDNA3.1/His/*lac*Z is included for mammalian cell transfection. **Do not** use this vector as a control for transfection into S2 cells. Use the positive control vector included with each DES™ Kit.

Item	Concentration	Amount supplied
Tissue Culture Sterile Water	–	2 × 12 mL
2X HEPES Buffered Saline (HBS)	50 mM HEPES 1.5 mM Na_2HPO_4 280 mM NaCl pH 7.1	2 × 12 mL
$CaCl_2$	2 M	3 × 1 mL
pcDNA™3.1/His/*lac*Z	40 µL at 0.5 µg/µL in TE, pH 8	20 µg (for mammalian transfection only)

Introduction

Product overview

Description of the system

The *Drosophila* Expression System (DES™) utilizes a cell line derived from *Drosophila melanogaster*, Schneider 2 (S2) cells, and a simple plasmid vector for the expression of heterologous proteins. S2 cells are easily maintained in loosely adherent or suspension culture at 26°C to 28°C, and they do not require CO_2.

A wide variety of vectors is available to allow expression of the recombinant protein of interest in S2 cells. Depending on your needs, the following expression vectors are available:

Vector	Benefit
pMT/V5-His	For inducible expression of recombinant protein from the metallothionein (MT) promoter (Bunch *et al.*, 1988; Maroni *et al.*, 1986)
pAc5.1/V5-His	For constitutive expression of recombinant protein from the actin (Ac5) promoter (Chung and Keller, 1990)
pMT/BiP/V5-His	For inducible expression of secreted proteins from a vector containing a *Drosophila* secretion signal
pMT/BioEase™-DEST	For inducible expression of biotinylated, recombinant proteins
pMT/V5-His-TOPO™	For rapid cloning of *Taq*-amplified PCR products and inducible expression of recombinant protein

Stable cell lines expressing heterologous proteins can be generated in 3–4 weeks from a single cotransfection of the expression vector and the pCoHygro selection vector or 2 weeks following cotransfection of the expression vector and the pCoBlast selection vector. By optimizing the ratio of expression vector to selection vector, cell lines with a very high copy number of the desired gene can be generated, leading to increased expression levels of the desired protein.

Continued on next page

Product overview, continued

Types of DES™ kits available

Many types of DES™ Kits are available for purchase, each containing a different set of expression vectors and either the pCoHygro or pCoBlast selection vector, as indicated in the table below. For more information about each expression vector, refer to the manual for the vector. Manuals are available for download from **www.thermofisher.com** or by calling Technical Support (see page 35).

Product	Expression vector	Selection vector	Catalog no.
DES™ Inducible Kit	pMT/V5-His A, B, and C	pCoHygro	K4120-01
		pCoBlast	K5120-01
DES™ Inducible/Secreted Kit	pMT/BiP/V5-His A, B, and C	pCoHygro	K4130-01
		pCoBlast	K5130-01
DES™ BioEase™ Expression Kit	pMT/BioEase™-DEST	pCoBlast	K4140-01
DES™ TOPO™ TA Expression Kit	pMT/V5-His-TOPO™	Not included	K4125-01

Experimental outline

The table below describes the basic steps needed to clone and express your protein using the DES™ kit of choice. For more details, refer to the pages indicated.

Step	Action	Page
1	Establish culture of S2 cells from supplied frozen stock.	4–7
2	Develop a strategy to clone your gene of interest into the desired vector in frame with the appropriate N- or C-terminal tag. For details, refer to the specific manual for each expression vector.	See vector manual
3	Once you have generated your expression construct, isolate purified, plasmid DNA and sequence your recombinant expression vector to confirm that your protein is in frame with the appropriate N- or C-terminal peptide, if desired.	See vector manual
4	Transiently transfect S2 cells.	9–14
5	Induce, if necessary, and assay for expression of your protein.	12
6	Create stable cell lines expressing the protein of interest by cotransfecting the recombinant expression vector with the selection vector, pCoHygro or pCoBlast, and selecting with the appropriate concentration of hygromycin-B or blasticidin, respectively.	15–18
7	Induce, if necessary, and assay for expression of your protein	18
8	Scale-up expression and purify your recombinant protein, if desired.	19–21

Methods

Culture of S2 cells

Introduction

The S2 cell line was derived from a primary culture of late stage (20–24 hours old) *Drosophila melanogaster* embryos (Schneider, 1972). Many characteristics of the S2 cell line suggest that it is derived from a macrophage-like lineage. S2 cells grow at 26°C to 28°C without CO_2 as a loose, semi-adherent monolayer in tissue culture flasks and in suspension in spinners and shake flasks.

Note: S2 cells may also be cultured at room temperature. However, temperature should be set to 26°C to 28°C to achieve robust growth and optimal doubling time of 18–24 hours.

General cell handling

- All solutions and equipment that come in contact with the cells must be sterile.
- Always use proper sterile technique in a laminar flow hood.
- All incubations are performed at 26°C to 28°C and do not require CO_2.

  Note: If you want to slow down S2 cell growth, you may incubate cells at room temperature (22–25°C).
- The complete medium for S2 cells is Schneider's *Drosophila* Medium containing 10% **heat-inactivated** fetal bovine serum (FBS). This medium is used for transient expression and stable selection. Schneider's *Drosophila* Medium and FBS are available separately for purchase.
- Adding antibiotics to media is not recommended; however, 5 mL/L of Penicillin-Streptomycin may be used when required.
- Before starting experiments, be sure to have established frozen S2 cell stocks that have been pre-qualified by testing for bacterial, fungal, and mycoplasma contamination as well as robust cell growth.
- Count cells before seeding for transfection or freezing cells for stocks. Check for viability (if desired) using trypan blue. S2 cell viability in culture should be >95%.
- Always use **new** flasks or plates when passing cells for general maintenance. During transfection and selection keep cells in the **same** culture vessel.
- S2 cells grow better if some conditioned medium is brought along when passaging cells.

  Note: Conditioned medium is medium in which cells have been grown.

Important

S2 cells do not completely adhere to surfaces, making it difficult to rinse the cells if needed. To exchange cells into new medium or to wash cells prior to lysis, follow the instructions below:

- Resuspend cells in the conditioned medium and centrifuge at 100 × *g* for 5–10 minutes. Decant the medium.
- Resuspend the cells in fresh medium (or PBS) and centrifuge as above. Repeat.
- Add fresh medium (or buffer) and replate the cells (or lyse them).

Continued on next page

Culture of S2 cells, continued

Materials required

Be sure to have the following solutions and supplies available :

- 15-mL sterile, conical tubes
- 5-, 10-, and 25-mL sterile pipettes
- Cryovials
- Reagents to count cells
- Complete Schneider's *Drosophila* Medium
- Heat-inactivated fetal bovine serum (FBS) (see page 33)

  Note: Make sure to use heat-inactivated FBS, because FBS that has not been heat-inactivated will inhibit the growth of S2 cells.
- Penicillin-Streptomycin (see page 33), if desired
- Table-top centrifuge
- T-25 cm^2 flasks, T-75 cm^2 flasks, T-175 cm^2 flasks, 35-mm plates, or 125-mL shake flasks (other flasks and plates may be used)
- Phosphate-Buffered Saline, pH 7.4 (PBS; see page 33)

Thaw cells for adherent culture

Store frozen S2 cells in liquid nitrogen until ready to use. Use the following procedure to thaw cells and initiate adherent cell culture. The vial of S2 cells contains 1×10^7 cells in 1 mL of Freezing Medium.

1. Remove the vial of cells from liquid nitrogen and thaw quickly in a 37°C water bath. Just before the cells are completely thawed, decontaminate the outside of the vial with 70% ethanol.
2. Gently transfer the entire contents of the cryovial into a sterile conical tube containing 30 mL of complete Schneider's *Drosophila* Medium at room temperature.

   Note: We do not recommend adding antibiotics to the complete medium used to initially culture thawed cells. Antibiotics may be added to the medium 24–48 hours after cells have been thawed.
3. Remove a small aliquot of cells to determine cell density and viability.
4. Transfer the ~30 mL of cell suspension into an appropriate size flask (T-175 cm^2 or greater), so that the seeding density falls between 1×10^4 to 5×10^4 viable cells per cm^2.
5. Place the flask in a 26°C to 28°C, non-humidified, ambient air-regulated incubator. Loosen the cap of the culture flask to allow oxygenation/aeration.
6. Once the culture has reached 80–90% confluency, subculture cells at a seeding density of 1×10^4 to 5×10^4 cells per cm^2 in complete Schneider's *Drosophila* Medium.

   Optional: Complete medium may contain antibiotics, if desired.

   Note: The S2 cell line is not a tightly adherent cell line. Cells that are floating in culture may not be dead. Check the cell viability using Trypan Blue or your method of choice.

Continued on next page

Culture of S2 cells, continued

Thaw cells for suspension culture

Store frozen S2 cells in liquid nitrogen until ready to use. Use the following procedure to thaw cells and initiate suspension cell culture. The vial of S2 cells contains 1×10^7 cells in 1 mL of Freezing Medium.

1. Remove the vial of cells from liquid nitrogen and thaw quickly in a 37°C water bath. Just before the cells are completely thawed, decontaminate the outside of the vial with 70% ethanol.
2. Gently transfer the entire contents of the cryovial into a sterile conical tube containing 27 mL of complete Schneider's *Drosophila* Medium at room temperature.
3. Remove a small aliquot of cells to determine cell density and viability.
4. Gently transfer the ~27 ml of cell suspension into a 125-mL Erlenmeyer flask and incubate on an orbital shaker with a shaking speed of 125–150 rpm in a 26°C to 28°C, non-humidified, ambient air-regulated incubator. Loosen the cap of the culture flask to allow for oxygenation/aeration.
5. Once the culture density has reached $>2.0 \times 10^6$ viable cells/mL, subculture cells at a density of 1×10^5 to 3×10^5 viable cells/mL in complete Schneider's *Drosophila* Medium at room temperature.

   Note: If cell density does not reach $>2.0 \times 10^6$ viable cells/mL in 4–5 days, centrifuge the cells at $100 \times g$ for 5 minutes and gently re-suspend in fresh complete growth medium.

Subculture adherent cells

For general maintenance, pass the cells when the adherent S2 culture reaches 80–90% confluence.

1. To remove cells from the flask, gently tap the flask several times to dislodge the cells that may be attached to the surface of the flask.
2. Use a 5-mL pipette to wash the surface of the flask with conditioned medium to remove the remaining adherent S2 cells. Transfer the cell suspension to a conical tube.
3. Remove 1 mL of the cell suspension from the conical tube to determine cell density and viability using your method of choice (e.g., hemocytometer and Trypan Blue).
4. Refer to the table below for adherent culture conditions of S2 cells in T-flasks.

Vessel size	Volume of media and cells	Seeding density	Temperature
T-75 cm^2 flask	12–15 mL	1×10^4 to 5×10^4 viable cells/cm^2	26°C to 28°C
T-175 cm^2 flask	40–50 mL		

Continued on next page

Culture of S2 cells, continued

Subculture suspension cells

For general maintenance, pass the cells when the suspension S2 culture reaches >1.0 × 10^7 viable cells/mL.

1. Remove 1 mL of the cell suspension from the stock flask to determine cell density and viability using your method of choice (e.g., hemocytometer and Trypan Blue).
2. Refer to the table below for suspension culture conditions of S2 cells.

Erlenmeyer flasks				
Vessel size	Vol. of media and cells	Seeding density	Shake speed	Temperature
125-mL Erlenmeyer	30–50 mL	1 × 10^5 to 3 × 10^5 viable cells/cm^2	125–150 rpm	26°C to 28°C
250-mL Erlenmeyer	75–100 mL			
>1 mL Erlenmeyer	one-fourth to one-third of the flask size			

Spinner flasks					
Vessel size	Vol. of media and cells	Seeding density	Passage density	Shake speed	Temperature
125-mL spinner	<75 mL	1 × 10^5 to 3 × 10^5 viable cells/cm^2	>1 × 10^7 viable cells/cm^2	See notes below	26°C to 28°C
250-mL spinner	<150 mL				

Notes:

1. The appropriate spinner or impeller speed and seeding density should be determined and optimized for each system. For spinner flasks >500 mL, use a vessel that provides for gas sparging (bubbling a chemically inert gas through a liquid).
2. The total culture volume should not exceed 60% of the indicated volume of spinner flask for proper aeration.
3. Determine the optimum impeller speed for your spinner vessel depending on your needs. To reduce loss of viability due to cell shearing, make sure that the impeller blade rotates freely and does not contact vessel walls or base.

Determine cell density and viability

Follow the procedure below to determine viable and total cell counts.

1. Transfer a small aliquot of the cell suspension to a microcentrifuge tube.
2. Determine viability using the trypan blue exclusion method.
3. Determine cell density electronically using a Coulter Counter or manually using a hemocytometer chamber.

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Freeze S2 cells

Label the appropriate number of cryovials before starting. Follow the recommended cell freezing conditions below.

1. Prepare the required volume of freezing medium composed of the following components:
   a. 46.25% fresh growth medium without antibiotics
   b. 46.25% conditioned growth medium (day 2 to 4 cell conditioned medium collected from S2 cultures during the subculture procedure)
   c. 7.5% DMSO

   Note: Prepare the freezing medium immediately before use. Filter-sterilize the freezing medium and chill at 4°C until use. Discard any remaining freezing medium after use.
2. Grow the desired quantity of S2 cells in shake, spinner, or T-flasks, and harvest them when the cells are in mid-log exponential growth and have a viability of ≥90%.
3. Determine viable and total cell counts and calculate the volume of freezing medium required to yield a final cell density of $\geq 1 \times 10^7$ viable cells/mL.
4. Centrifuge the cells at 100 × *g* for 5 minutes. Aseptically decant the supernatant and resuspend the cell pellet in the pre-determined volume of chilled freezing medium**.**
5. Dispense aliquots of the cell suspension (frequently mixing to maintain a homogeneous cell suspension) into cryovials according to manufacturer's specifications (*i.e.* 1.5 mL in a 2-mL cryovial).
6. Freeze the cells in an automated, controlled-rate freezing apparatus or using a manual method following standard procedures in a –70°C freezer. For ideal cryopreservation, the freezing rate should be a decrease of 1°C per minute.
7. Transfer the frozen vials to liquid nitrogen (vapor phase) for long-term storage.

   Note: We recommend that you pre-qualify the cryopreserved cells before use. Thaw one vial of the cryopreserved cells 24 hours after storing the vials in liquid nitrogen by following the procedure outlined in **Thawing cells**, page 4. Test the cells for growth, morphology, and bacterial, fungal, and mycoplasma contamination. The cell growth rate and morphology should look the same as the stock culture that you should continue growing until the cell freeze has been qualified.

Propagation and maintenance of plasmids

Introduction

The following section contains guidelines for maintaining and propagating the pCoHygro and pCoBlast vectors. For information about maintaining and propagating the DES™ expression vectors, refer to the manual for the vector you are using.

General molecular biology techniques

For help with *E. coli* transformations, restriction enzyme analysis, and plasmid preparation, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

E. coli host

Many *E. coli* strains are suitable for the propagation of the pCoHygro and pCoBlast vectors. We recommend that you propagate the vectors in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A) such as TOP10 or DH5α™-T1^R. TOP10 and DH5α™-T1^R *E. coli* are available as chemically competent or electrocompetent (TOP10 only) cells in a One Shot™ format for purchase (see page 33).

Transformation method

You may use any method of choice for transformation. Chemical transformation is the most convenient for many researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintain plasmids

The pCoHygro and pCoBlast vectors contain the ampicillin gene for selection using ampicillin (see pages 30–32 for more information about the vectors). To propagate and maintain each plasmid:

1. Use the supplied 0.5 µg/µL stock solution in TE buffer, pH 8.0 to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™-T1^R, or equivalent.
2. Select transformants on LB agar plates containing 100 µg/mL ampicillin. For fast and easy microwaveable preparation of Low Salt LB agar containing ampicillin, imMedia™ Amp Agar is available for purchase (see page 33). For more information, call Technical Support (see page 35).
3. Prepare a glycerol stock of each plasmid for long-term storage (see page **Error! Bookmark not defined.**).

Prepare a glycerol stock

Once you have identified the correct clone, purify the colony and make a glycerol stock for long-term storage. We also recommend keeping a stock of your plasmid DNA at –20°C.

1. Streak the original colony out on an LB plate containing 100 µg/mL ampicillin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 10 µg/mL ampicillin.
3. Grow the culture to mid-log phase (OD_{600} = 0.5–0.7).
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Transient transfection of S2 cells

Introduction

Drosophila S2 cells may be transfected with the recombinant expression vector alone to assay for transient expression of the protein of interest (below) or in combination with the selection vector, pCoHygro or pCoBlast, to generate stable cell lines (see page 15). We recommend that you test for expression of your recombinant protein by transient transfection before undertaking selection for stable cell lines. The method of choice for transfection of S2 cells is calcium phosphate.

Generate the expression construct

A separate manual is provided to facilitate cloning your gene of interest into the DES™ expression vector. For instructions, maps of the vector, and other details, refer to the specific manual for each vector. The complete sequence for each DES™ expression vector is available for download from **www.thermofisher.com** or by calling Technical Support (see page 35). Once you have obtained your expression construct, prepare purified plasmid DNA for transfection into S2 cells.

Plasmid preparation

Plasmid DNA for transfection into S2 cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HiPure Miniprep Kit or CsCl gradient centrifugation. The PureLink™ HiPure MidiPrep Kit is a medium-scale plasmid isolation kit that isolates up to 150 µg of plasmid DNA from 25–100 mL of bacterial culture. Plasmid can be used directly for transfection of S2 cells.

Positive control

We recommend that you include a positive control as well as a negative control (empty vector) in your transfection experiment to help you evaluate your results. A control expression plasmid is included with each DES™ kit (see table below) to help you optimize transfection and expression conditions. See the expression vector manual for more information on the positive control vector supplied with each expression vector.

Product	Expression vector	Control vector
DES™ Inducible Kit	pMT/V5-His A, B, and C	pMT/V5-His/*lac*Z
DES™ Inducible/Secreted Kit	pMT/BiP/V5-His A, B, and C	pMT/BiP/V5-His/GFP
DES™ BioEase™ Expression Kit	pMT/BioEase™-DEST	pMT/BioEase™-GW/*lacZ*
DES™ TOPO™ TA Cloning Kit	pMT/V5-His-TOPO™	pMT/*lacZ*

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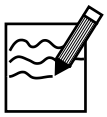
Transient transfection of S2 cells, continued

Important

The first time you perform a transient transfection you may wish to perform a time course to ensure that you detect expression of your protein. We suggest assaying for expression at 2, 3, 4, and 5 days posttransfection.



Note

Transient and stable transfections may be set up in side-by-side experiments for efficiency. If expression is detected from the transient transfection, you may proceed directly with selection of polyclonal cell lines.

Before starting

Be sure and have the following reagents and equipment ready before starting:

- S2 cells growing in culture (You will need 3×10^6 S2 cells per 35-mm plate per transfection)
- 35-mm plates (other flasks or plates can be used, see page 25)
- Complete Schneider's *Drosophila* Medium (see page 3)
- Recombinant plasmid DNA (19 µg per transfection; may be varied for optimum expression)
- Sterile microcentrifuge tubes (1.5 mL)
- Calcium Phosphate Transfection Kit (included with all DES™ Kits except the DES™ TOPO™ TA Cloning Kit; Catalog no. K2780-01)
- 100 mM copper sulfate (if needed; supplied with DES™ Kits containing an inducible expression vector)
- PBS, pH 7.4 (see page 33 for ordering information)
- Cell Lysis Buffer (see recipe on page 22)

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Transient transfection of S2 cells, continued

Calcium phosphate transfection procedure

Use the procedure below to transiently transfect S2 cells. Instructions are for one transfection in a 35-mm plate. You may want to include additional plates for time points after transfection. We recommend that you test for expression of your protein before selecting for a stable population.

Day 1: Preparation

1. Prepare cultured cells for transfection by seeding 3×10^6 S2 cells in a 35-mm plate in 3 mL complete Schneider's *Drosophila* Medium (1×10^6 cells/mL).
2. Grow 6 to 16 hours at 28°C until cells reach a density of 2×10^6–4×10^6 cells/mL.

Day 2: Transient transfection

3. Prepare the following transfection mix (per 35-mm plate). **Note:** You do not need the selection vector, pCoHygro or pCoBlast, for transient transfections.

   In a microcentrifuge tube mix together the following components. This will be **Solution A**.

2 M $CaCl_2$	36 µL
Recombinant DNA (19 µg)	X µL
Tissue culture sterile water	Bring to a final volume of 300 µL

   In a second microcentrifuge tube, add 300 µL 2X HEPES-Buffered Saline (HBS; 50 mM HEPES, 1.5 mM Na_2HPO_4, 280 mM NaCl, pH 7.1). This is **Solution B**.
4. Slowly add **Solution A** dropwise to **Solution B** with continuous mixing (you may vortex or bubble air through the solution). Continue adding and mixing until **Solution A** is depleted. This is a slow process (1–2 minutes). Continuous mixing ensures production of the fine precipitate necessary for efficient transfection.
5. Incubate the resulting solution at room temperature for 30–40 minutes. After ~30 minutes a fine precipitate should form.
6. Mix the solution and add dropwise to the cells. Swirl to mix in each drop.
7. Incubate 16 to 24 hours at 28°C. **Note**: You may wish to investigate whether extending the incubation time improves transfection efficiency.

Day 3: Posttransfection

8. Remove calcium phosphate solution and wash the cells twice with complete medium. To wash cells, resuspend cells in complete medium and centrifuge at $100 \times g$ for 5 to 10 minutes. Decant the medium. Add fresh medium and replate into the same vessel. Continue to incubate at 28°C.
9. Induce expression when the cells either reach log phase ($2–4 \times 10^6$ cells/mL) or 1 to 4 days after transfection. Add copper sulfate to the medium to a final concentration of 500 µM. Induce for 24 hours before assaying protein.

Day 4+: Harvesting cells

10. Harvest the cells 2, 3, 4, and 5 days posttransfection and assay for expression of your gene (see page 12). Addition of fresh medium or more inducer is not necessary.

Continued on next page

Note

The presence of N-terminal or C-terminal tags or the secretion signal will increase the size of your protein. Refer to the table below for the approximate size of each peptide. Note that any additional amino acids between your protein and the tags are not included. Note also that the secretion signal should be cleaved from your protein upon secretion, hence the you should not see an increase in the molecular weight of your protein when running on a gel.

Peptide	Molecular Weight (kDa)
BiP secretion signal	1.8
V5-His C-terminal tag	2.6
BioEase™ tag	9.8

Detection of recombinant fusion proteins

Depending on the expression vector you are using, you may detect expression of your recombinant fusion protein by western blot analysis using reagents available for purchase (see pages 33–34 for ordering information).

- For recombinant fusion proteins containing the C-terminal V5-His tag, you may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies.
- For recombinant fusion proteins containing the N-terminal BioEase™ tag, you may use the Streptavidin conjugates.

For more information, refer to **www.thermofisher.com** or call Technical Support (see page 35).

Test for expression

Use the cells from one 35-mm plate for each expression experiment.

1. Prepare an SDS-PAGE gel or use a pre-cast gel (see page 13) that will resolve your expected recombinant protein.
2. Transfer cells to a sterile, 1.5 mL microcentrifuge tube. **If you are using pMT/BiP/V5-His to express your protein, be sure to save and assay the medium.**
3. Pellet cells at 100 × *g* for 5 to 10 minutes. Transfer the supernatant (medium) to a new tube and resuspend the cells in 1 mL PBS.
4. Pellet cells and resuspend in 50 µL Cell Lysis Buffer (see page 22 for a recipe). Other recipes are suitable. Vortex.
5. Incubate the cell suspension at 37°C for 10 minutes. **Note:** You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
6. Vortex and pellet nuclei and cell debris. Transfer the supernatant to a new tube, and assay the lysate for protein concentration.
7. Add SDS-PAGE sample buffer (see page 22 for a recipe) to a final concentration of 1X and boil the sample for 5 minutes.
8. Load 3 to 30 µg of lysate onto an SDS-PAGE gel and electrophorese. Amount loaded depends on the amount of your protein produced. Load varying amounts of lysates or medium.

Continued on next page

Transient transfection of S2 cells, continued

Polyacrylamide gel electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE™ and Novex™ Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available for purchase. In addition, we also carry a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to **www.thermofisher.com** or call Technical Support (see page 35).

Assay for β-galactosidase

If you use an expression control containing *lacZ* in your experiment (e.g. pMT/V5-His/*lac*Z or pMT/BioEase™-GW/*lacZ*), you may assay for β-galactosidase expression by activity assay using cell-free lysates (Miller, 1972). We offer the β-Gal Assay Kit and the β-gal Antiserum for fast and easy detection of β-galactosidase expression (see page 33 for ordering information).

Assay for Cycle 3-GFP

If you use pMT/BiP/V5-His/GFP (cycle 3-GFP) as a positive control vector, you may assay for GFP expression in the following ways:

- Use fluorescence microscopy to visualize GFP-expressing cells

  To detect fluorescent cells, it is important to pick the best filter set to optimize detection. The primary excitation peak of cycle 3-GFP is at 395 nm. There is a secondary excitation peak at 478 nm. Excitation at these wavelengths yield a fluorescent emission peak with a maximum at 507 nm.

- Use fluorescence spectroscopy to assay the medium

  You can detect cycle 3-GFP fluorescence in the medium using fluorescence spectroscopy. Be sure to run a mock sample (medium alone) as the Schneider's *Drosophila* Medium has some autofluorescence (Zylka and Schnapp, 1996) that must be subtracted as background.

- Use western blot analysis to assay for GFP protein

  GFP Antiserum is available for purchase (see page 33).

After transfection, allow the cells to recover for 24 to 48 hours before inducing expression of cycle 3-GFP with copper sulfate. Induce for ~20 hours before assaying for fluorescence. For more details about detection of fluorescence, refer to the pMT/BiP/V5-His manual.

Continued on next page

Troubleshooting

Cells growing too slowly (or not at all)

- Cells were seeded at too low of a density during subculturing. Do not seed cells at less than 5×10^5 viable cells/mL. Cells will eventually grow back up if they were not seeded at too low of a density. If cells do not seem to be growing, thaw a new vial of cells.
- Cells grow better if conditioned medium is used throughout passaging.

Low transfection efficiency

If you feel your transfection efficiencies are too low, check the following:

- Use pure plasmid DNA isolated using the PureLink™ HiPure MidiPrep Kit or CsCl gradient ultracentrifugation.
- Make sure the calcium phosphate precipitate is fine enough. Be sure to thoroughly and continuously mix Solution B while you are adding Solution A.

Low or no protein expression

- **Gene not cloned in-frame with signal sequence or appropriate N- or C-terminal tag.** If your protein is not in frame with the BiP signal sequence, it will not be expressed or secreted. If it is not in frame with the appropriate N- or C-terminal tag, expression will not be detected using the appropriate reagent (e.g. V5 antibody or streptavidin conjugate).
- **No Kozak sequence for proper initiation of transcription.** Translation will be inefficient and the protein will not be expressed at its optimal level.
- **Gene product is toxic to S2 cells.** Use one of the inducible DES™ vectors (*i.e.* pMT/V5-His, pMT/BiP/V5-His, or pMT/BioEase™-DEST).

Selection of stable cell lines

Introduction

Once you have demonstrated that your protein is expressed in S2 cells, you may wish to create stable cell lines for increased expression or large-scale production of the desired protein. *Drosophila* stable cell lines generally contain multicopy inserts that form arrays of more than 500–1000 copies in a head to tail fashion (Kirkpatrick and Shatzman, 1997). The number of inserted gene copies can be manipulated by varying the ratio of expression and selection plasmids. We recommend using a 19:1 (w/w) ratio of expression vector to selection vector. You may vary the ratios to optimize expression of your particular gene.

Selection vectors

The DES™ kits are generally available with a choice of selection vector, pCoHygro or pCoBlast. The pCoHygro and pCoBlast vectors use the *copia* promoter to control expression of the hygromycin (*HPH*) (Gritz and Davies, 1983) or blasticidin (*bsd*) (Kimura *et al.*, 1994) resistance genes, respectively. You will cotransfect your expression vector with pCoHygro or pCoBlast into S2 cells to generate stable cell lines. For more information about each selection vector, refer to pages 30–32.

Using Hygromycin or Blasticidin

After cotransfection of your expression vector and pCoHygro or pCoBlast, you will use the hygromycin B or blasticidin selection agents, respectively, to select for stable transfectants. The DES™ kits containing pCoHygro include hygromycin B, while the DES™ kits containing pCoBlast include blasticidin to facilitate selection of stable cell lines. For more information about preparing and using hygromycin and blasticidin, refer to the **Appendix**, pages 23 and 24.

We recommend using Schneider's *Drosophila* Medium to select stable S2 cell lines with hygromycin or blasticidin. Once stable cell lines have been generated, cells may be maintained in Schneider's *Drosophila* Medium containing the appropriate concentration of antibiotic.

It may be possible to use serum-free medium to select stable S2 cell lines; however, some serum-free media can **only** be used for recombinant protein expression and purification purposes and **cannot** be used for hygromycin or blasticidin selection. Addition of hygromycin or blasticidin to some serum-free media will kill S2 cells, even those that are hygromycin-resistant or blasticidin-resistant in serum-containing medium, respectively. If you want to use serum-free medium for selection, we suggest that you test your serum-free medium directly.

Continued on next page

Selection of stable cell lines, continued

Hygromycin-B selection guidelines

To select for S2 cells that have been stably cotransfected with pCoHygro and a DES™ expression vector, we generally use 300 µg/mL of hygromycin-B. If this concentration does not work for you, we recommend that you perform a kill curve as described below. Hygromycin activity may vary from lot to lot.

- Prepare complete Schneider's *Drosophila* Medium supplemented with 100–1000 µg/mL hygromycin-B.
- Test varying concentrations of hygromycin-B on the S2 cell line to determine the concentration that kills your cells (kill curve).
- Calculate concentration based on the amount of active drug (check the lot label).

Cells will divide once or twice in the presence of lethal doses of hygromycin, so the effects of the drug take several days to become apparent. Complete inhibition of cell growth can take 3–4 weeks of growth in selective medium. Cell death can be verified by trypan blue staining.

Blasticidin selection guidelines

To select for S2 cells that have been stably cotransfected with pCoBlast and a DES™ expression vector, we generally use 25 µg/mL of blasticidin. If this concentration does not work for you, we recommend that you perform a kill curve as described below.

- Prepare complete Schneider's *Drosophila* Medium supplemented with 5-100 µg/mL blasticidin.
- Test varying concentrations of blasticidin on the S2 cell line to determine the concentration that kills your cells (kill curve).

Selection with blasticidin is generally much faster than selection with hygromycin. We typically observe complete inhibition of cell growth after 2 weeks in selective medium. Cell death can be verified by trypan blue staining.

Before starting

Be sure and have the following reagents and equipment ready before starting:

- S2 cells growing in culture
- 35-mm plates (other flasks or plates can be used)
- Complete Schneider's *Drosophila* Medium (see page 3)
- Hygromycin-B or blasticidin (included in the appropriate kit)
- Recombinant DNA (19 µg per transfection)
- pCoHygro or pCoBlast (1 µg/µL solution in sterile water or TE; use 1 µg per transfection)
- Sterile microcentrifuge tubes (1.5 mL)
- Cell Lysis Buffer (see recipe on page 22)
- Calcium Phosphate Transfection Kit (included with the kit)

Continued on next page

Stable transfection using Calcium Phosphate

Follow this procedure to stably transfect cells. Use a ratio of 19:1 (Expression vector:Selection vector). To optimize expression of your protein, you may vary the ratio of expression vector to selection vector used. Include a negative control (empty vector) and a positive control (included with the DES™ kit of choice).

Day 1: Preparation

1. Seed 3×10^6 S2 cells in a 35-mm plate in 3 mL complete growth medium.
2. Grow 6 to 16 hours at 28°C until the cells reach log phase (2×10^6–4×10^6 cells/mL).

Day 2: Transfection

3. Prepare the following transfection mix for a 35-mm plate:

   In a microcentrifuge tube, mix together the following components. This will be **Solution A**.

2 M $CaCl_2$	36 µL
Recombinant DNA (19 µg)	X µL
pCoHygro or pCoBlast (1 µg)	2 µL
Tissue culture sterile water	Bring to a final volume of 300 µL

   To a second microcentrifuge tube add 300 µL 2X HBS. This is **Solution B**.
4. Slowly add **Solution A** dropwise to **Solution B** with continuous mixing. Continue adding and mixing until **Solution A** is depleted.
5. Incubate the resulting solution at room temperature for 30–40 minutes. After ~30 minutes a fine precipitate will form.
6. Mix the solution and add dropwise to the cells. Swirl the plate to mix in each drop after it is added. Incubate for 16–24 hours at 28°C.

Day 3: Posttransfection

7. Remove the calcium phosphate solution and wash the cells twice with complete medium. To wash cells, resuspend cells in complete medium and centrifuge at 100 × *g* for 5–10 minutes. Decant the medium. Add fresh complete medium **(no selection agent)** and replate into the same well or plate. Do not split cells.
8. Incubate at 28°C for 2 days.

Day 5: Selection

9. Centrifuge the cells and resuspend in complete growth medium containing the appropriate concentration of antibiotic. Replace selective medium every 4 to 5 days until resistant colonies appear (3–4 weeks for hygromycin-B; 2 weeks for blasticidin).

+3 Weeks: Expansion

10. Replate resistant clones into new plates containing **selective** medium, and pass cells when they reach a density of 6×10^6–20×10^6 cells/mL. This is to remove dead cells. **Note**: You may plate resistant cells into smaller plates or wells to promote cell growth before expansion for large-scale expression or preparation of frozen stocks.
11. Expand resistant cells into 6-well plates to test for expression or into flasks to prepare frozen stocks. **Maintain cells in selective medium**.

Continued on next page

Selection of stable cell lines, continued

Assay for expression

Induce, if necessary, and assay each of your stable cell lines for yield of the desired protein and select the one with the highest yield for scale-up and purification of recombinant protein. **If your protein is secreted, remember to assay the medium.** You may wish to compare the yield of protein in the cells and supernatant.

See page 11 for information on induction with copper sulfate.

Using different inducers

Other researchers have used 10 µM $CdCl_2$ to induce the metallothionein promoter (Johansen *et al.*, 1989). While cadmium is an effective inducer, be aware that cadmium will also induce a heat shock response in *Drosophila*.

In addition, higher concentrations of copper sulfate (600 µM to 1 mM) have been used to induce some proteins (Millar *et al.*, 1994; Tota *et al.*, 1995; Wang *et al.*, 1993).

Remember to prepare master stocks and working stocks of your stable cell lines prior to scale-up and purification.

Scale-up and purification

Introduction

Once you have obtained stable cell lines expressing the protein of interest and prepared frozen stocks of your cell lines, you are ready to scale-up expression and purify your protein. General information for protein purification is provided below. If your protein is secreted, you may want to culture cells in serum-free medium to simplify purification (see page 20).

Scale-up

To scale up S2 cell culture, refer to the table on page 25 for the recommended volumes to use in various culture vessels. On page 26 is a protocol for growing cells in suspension culture in either spinners or shake flasks.

Expressing heterologous proteins

A large number of proteins have been expressed using *Drosophila* S2 cells. A table listing some of these proteins is provided in the **Appendix**, pages 27–29.

Purification reagents

Depending on the expression vector you are using, reagents are available for purchase to facilitate purification of recombinant fusion proteins.

- If your recombinant fusion protein contains the C-terminal V5-His peptide, the presence of the 6xHis tag allows you to purify your protein using a metal-chelating resin such as ProBond™ or Ni-NTA (see below for more information).
- If your recombinant fusion protein contains the N-terminal BioEase™ tag, you may take advantage of the strong association between biotin and avidin (and its analogs including streptavidin) to purify your biotinylated protein using streptavidin agarose-conjugated beads (see page 20 for more information).

ProBond™ and Ni-NTA

ProBond™ and Ni-NTA are nickel-charged agarose resins that can be used for affinity purification of fusion proteins containing the 6xHis tag. Proteins bound to the resin may be eluted with either low pH buffer or competition with imidazole. To purify your fusion protein using ProBond™ or Ni-NTA, refer to the manual included with each product. You may download the manuals from **www.thermofisher.com**.

Note

Many *Drosophila* proteins are naturally rich in histidines, with some containing stretches of six histidines. If you use a metal-chelating resin to purify your recombinant protein, these histidine-rich proteins may co-purify with your protein of interest. The contamination can be significant if your protein is expressed at low levels. We recommend adding 5 mM imidazole to the binding buffer prior to addition of the protein mixture to the column. Addition of imidazole may help to reduce background contamination by preventing proteins with low specificity from binding to the metal-chelating resin.

Continued on next page

Streptavidin-agarose beads

The streptavidin-agarose resin (see page 33 for ordering information) can be used for affinity purification of recombinant fusion proteins containing the BioEase™ tag, and is constructed by covalently linking streptavidin to cross-linked agarose beads via a 15-atom hydrophilic spacer arm specifically designed to reduce non-specific binding and ensure optimal binding of biotinylated molecules. For guidelines to purify your fusion protein using streptavidin-agarose, refer to the Streptavidin-Agarose manual, which is available for download from **www.thermofisher.com** or by calling Technical Support (see page 35).

Important

Because of the extremely strong interaction between streptavidin and biotin, the recombinant fusion protein must be eluted from streptavidin-agarose resin by cleavage using an enterokinase. We generally use EKMax™ (see page 33 for ordering information) and incubate overnight. For more information, refer to the Streptavidin-Agarose manual.

Serum-free medium

It is possible to maintain hygromycin-resistant or blasticidin-resistant S2 cells in serum-free medium for expression and purification purposes. Note that addition of hygromycin or blasticidin to some serum-free media can kill even hygromycin- or blasticidin-resistant S2 cells, so you will need to test your serum-free medium directly. We have routinely maintained hygromycin-resistant or blasticidin-resistant S2 cells in serum-free medium lacking antibiotic for up to a week.

If you are culturing cells in serum-free medium and plan to use a metal-chelating resin (e.g. ProBond™ or Ni-NTA) to purify your secreted protein, **note that adding serum-free medium directly to the column will strip the nickel ions from the resin.** See **Purification of 6xHis-Tagged Proteins from Medium**, page 21 for a general recommendation to address this issue.

Purify proteins from medium

Many protocols are suitable for purifying proteins from the medium. The choice of protocol depends on the nature of the protein being purified. Note that the culture volume needed to purify sufficient quantities of protein is dependent on the expression level of your protein and the method of detection. To purify 6xHis-tagged proteins from the medium, see the page 21.

Continued on next page

Purification of 6xHis-tagged proteins from medium

To purify 6xHis-tagged recombinant proteins from the culture medium, we recommend that you perform ion exchange chromatography prior to affinity chromatography on metal-chelating resins. Ion exchange chromatography allows:

- Removal of media components that strip Ni^{+2} from metal-chelating resins
- Concentration of your sample for easier manipulation in subsequent purification steps

Conditions for successful ion exchange chromatography will vary depending on the protein. For more information, refer to *Current Protocols in Molecular Biology*, Unit 10 (Ausubel *et al.*, 1994) or the *Guide to Protein Purification* (Deutscher, 1990).

Note: If you do not wish to perform ion exchange chromatography, you may also dialyze your sample prior to purification on metal-chelating resin. Dialysis will not concentrate your sample.

Remove the N-terminal BioEase™ tag with enterokinase

The pMT/BioEase™-DEST vector contains an enterokinase (EK) recognition site to allow removal of the BioEase™ tag from your recombinant fusion protein, if desired. A recombinant preparation of the catalytic subunit of bovine enterokinase (EKMax™) is available to remove the BioEase™ tag from your recombinant protein (see page 33 for ordering information). Instructions for digestion are included with the product. For more information, see **www.thermofisher.com/support** or call Technical Support (see page 35).

Appendix

Recipes

Cell lysis buffer

50 mM Tris, pH 7.8

150 mM NaCl

1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions. For 100 mL, combine

1 M Tris base	5 mL
5 M NaCl	3 mL
Nonidet P-40	1 mL

2. Bring the volume up to 90 mL with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 mL. Store at room temperature.

Note: Just before use, you may add protease inhibitors to a small volume of lysis buffer at the following final concentrations:

1 mM PMSF

1 µg/mL pepstatin

1 µg/mL leupeptin

4X SDS-PAGE sample buffer

Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	5 mL
Glycerol (100%)	4 mL
β-mercaptoethanol	0.8 mL
Bromophenol Blue	0.04 g
SDS	0.8 g

Yield is ~10 mL .

Aliquot and freeze at –20°C until needed.

Hygromycin

Hygromycin B

The pCoHygro selection vector contains the *E. coli* hygromycin resistance gene (*HPH*) (Gritz and Davies, 1983) for selection of transfectants with the antibiotic, hygromycin B (Palmer *et al.*, 1987). When added to cultured *Drosophila* cells, hygromycin B acts as an aminocyclitol to inhibit protein synthesis by disrupting translocation and promoting mistranslation.

Handling Hygromycin B

- Hygromycin B is light sensitive. Store the liquid stock solution at 4°C protected from exposure to light.
- Hygromycin is toxic. Do not ingest solutions containing the drug.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling hygromycin B and hygromycin B-containing solutions.

Preparing and storing Hygromycin B

Hygromycin B is supplied in the DES™ Kits containing pCoHygro, but may also be obtained separately in 1 gram aliquots (see page 33 for ordering information). The hygromycin B (MW = 527.5) included with the DES™ kits is supplied as a 100 mg/mL stock solution in autoclaved, deionized water and is filter-sterilized. The solution is brown in color. The stability of hygromycin B is guaranteed for six months, if stored at 4°C. Medium containing hygromycin B is stable for up to six weeks.

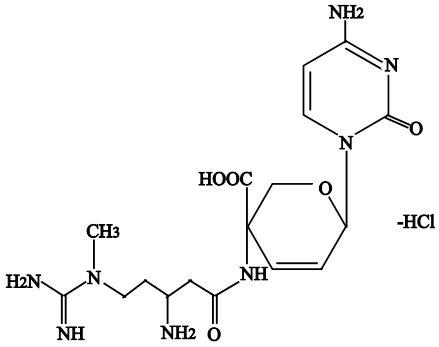
Blasticidin

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two blasticidin S deaminase genes: *bsd from Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Molecular weight, formula, and structure

The formula for blasticidin S is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of blasticidin.



Handling Blasticidin

Wear gloves, mask, goggles, and protective clothing (e.g. a laboratory coat) when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.

Preparing and storing stock solutions

Blasticidin is supplied in the DES™ Kits containing pCoBlast, but may also be obtained separately in 50 mg aliquots (see page 33 for ordering information). Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5 to 10 mg/mL.

- Dissolve blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use and freeze at –20°C for long-term storage or store at 4°C for short-term storage.
- Aqueous stock solutions are stable for 1–2 weeks at 4°C and 6–8 weeks at –20°C.
- pH of the aqueous solution should be 7.0 to prevent inactivation of blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store the thawed stock solution at 4°C for up to 2 weeks.
- Medium containing blasticidin may be stored at 4°C for up to 2 weeks.

Culture volumes

Table

The table below describes the optimal culture volume for use with a variety of culture vessels. The optimal volume is the volume needed for ideal surface area. The maximum volume is the volume needed for sustained growth. N/A--not applicable (Kirkpatrick and Shatzman, 1997). **Note**: We have found that you can increase the rpm in spinner flasks to prevent clumping.

Vessel	Optimal volume	Maximum volume	Optimal RPM	Caps
96-well plate	50 µL/well	200 µL	N/A	Sealed with Parafilm™
24-well plate	300 µL/well	600 µL	N/A	Sealed with Parafilm™
12-well plate	600 µL/well	1 mL	N/A	Sealed with Parafilm™
6-well plate	1.5 mL/well	2.5 mL	N/A	Sealed with Parafilm™
25-cm^2 flask	5 mL	N/A	N/A	Loosened
75- cm^2 flask	15 mL	N/A	N/A	Loosened
150-cm^2 flask	30 mL	N/A	N/A	Loosened
125-mL spinner	70 mL	100 mL	100	Loosened (¼ turn)
250-mL spinner	120 mL	150 mL	100	Loosened
500-mL spinner	250 mL	300 mL*	80–90	Loosened
1000-mL spinner	400 mL	600 mL	70–80	Loosened
3000-mL spinner	800 mL	1000 mL	70–80	Loosened
250-mL shake flask	100 mL	150 mL	115	Loosened
500-mL shake flask	200 mL	300 mL	115	Loosened
1000-mL shake flask	500 mL	700 mL	115	Loosened
3000-mL shake flask	1000 mL	1200 mL	115	Loosened

*Volume may be increased to 500 mL in Bellco spinner flasks.

Suspension cell cultures

Introduction

For large-scale growth and purification, S2 cells can be grown in suspension culture. Use the following protocol as a starting point for scale-up. This protocol can be easily adapted to shake flasks, if desired.

Materials needed

Be sure to have the following reagents on hand before starting:

- S2 cells in culture (either adherent or in suspension)
- Pluronic F-68, if desired (see page 33 for ordering information)
- Heparin (Sigma, Ca. no. H3149)
- Complete Schneider's *Drosophila* Medium
- 250-mL spinner flask (other flasks may be used)
- Magnetic stir plate

Before starting

Optional: You may want to add Pluronic F-68 to the medium at a concentration of 0.05–0.1%. Pluronic F-68 is a surfactant that prevents the cells from shearing.

Cell clumping

Cell clumping is more likely to occur in medium containing serum. If cell clumping (>10 cells per clump) occurs, add heparin at 10 Units/mL. Clumping occurs at the higher cell densities (*i.e.* > 8×10^6 cells/mL in serum containing medium and >30×10^6 cells/mL in serum-free medium).

Hygromycin

Stable cell lines can be grown in large-scale production without hygromycin-B. If you elect to use hygromycin-B, note that the stability will decrease at room temperature. Add hygromycin-B only as needed to the cell culture.

Procedure

1. For a culture volume of 125–150 mL use a 250-mL spinner flask. Usually, the culture volume should be about half the total vessel volume (capacity).
2. Inoculate spinner flask with either serum-free or serum containing medium and seed cells at approximately 1–2 × 10^6 cells/mL and viability of >95%.
3. Incubate spinner at 22–24°C with a constant stirring rate of 90–125 rpm. Loosen the side arms approximately a quarter (¼) turn. Increase rate to 140 rpm when cell densities reach >10×10^6 cells/mL.
   - Cell viability begins to decrease at densities >1.2×10^7 cells/mL in medium containing serum. Keep cell densities between 5-10 × 10^6 cells/mL in serum-containing medium.
   - Cell densities can approach 3×10^7 cells/mL without significant decrease in viability in serum-free medium.
4. Subculture cells to ~5×10^6 cells/mL when densities reach about ~1×10^7 cells/mL.

   Note: If cells are subcultured at densities below 1×10^6 cells/mL, the growth rate will significantly decrease.

Proteins expressed using *Drosophila* S2 cells

Table

Drosophila S2 cells have been used to express proteins for both biochemical and biological assays. The following table provides a representative list of proteins which have been expressed using the *Drosophila* S2 cells. The table includes information about posttranslational modifications, the expression level reported, and the reference.

Protein	Posttranslational modifications	Expression level	Reference
Enzymes			
Human dopamine β-hydroxylase	secreted, glycosylated	>16 mg/liter	(Li *et al.*, 1996)
Human plasminogen	secreted	10–15 mg/liter	(Nilsen and Castellino, 1999)
Viral proteins			
HIV-1 gp120	secreted, glycosylated	2 mg/liter	(Culp *et al.*, 1991)
HIV-1 gp160	secreted, glycosylated, proteolytic processing	Not reported	(Brighty and Rosenberg, 1994; Ivey-Hoyle and Rosenberg, 1990)
HIV-1 Rev regulatory protein	Not reported	Not reported	(Ivey-Hoyle and Rosenberg, 1990)
Antibodies			
Human IgG_1	secreted	> 1 mg/liter	(Kirkpatrick *et al.*, 1995)
Mouse single-chain variable fragment (scFv) from monoclonal antibody against African cassava mosaic virus (ACMV)	secreted	20 mg/liter	(Reavy *et al.*, 2000)
Cytokines			
Human Interleukin 5 (IL5)	Dimer, secreted, glycosylated, disulfide bonds	22 mg/liter	(Johanson *et al.*, 1995)
Human Interleukin 12 (IL12)	Heterodimer, secreted, glycosylated, disulfide bonds	10 mg/liter	(Lehr *et al.*, 2000)

Continued on next page

Proteins expressed using *Drosophila* S2 cells, continued

Table, continued

Protein	Posttranslational modifications	Expression level	Reference
Receptors			
Human IL5 Receptor α chain (membrane-bound and soluble forms)	membrane protein	17 and 10 mg/liter, respectively 1×10^6 sites/cell	(Johanson *et al.*, 1995)
Human Erythropoietin Receptor	secreted	5 mg/liter	(Lehr *et al.*, 2000)
Human Glucagon Receptor	glycosylation membrane protein	250 pmoles/mg membrane protein	(Tota *et al.*, 1995)
MHC class II I-E^d αβ heterodimers		0.1 – 0.4 mg/liter	(Wallny *et al.*, 1995)
Drosophila Muscarinic Acetylcholine Receptor	glycosylation membrane protein	2.4 pmoles/mg membrane protein	(Millar *et al.*, 1995)
Drosophila GABA Receptor	membrane protein	2.7 pmoles/mg membrane protein ~35,000 sites/cell	(Millar *et al.*, 1994)
S. calcitrans STKR G-protein coupled receptor	Not reported	Not reported	(Torfs *et al.*, 2000)
Rat calcitonin receptor-like receptor	glycosylation	Not reported	(Aldecoa *et al.*, 2000)
Cell adhesion proteins			
Drosophila Notch, Delta	Not reported	Not reported	(Fehon *et al.*, 1990)
Drosophila Chaoptin	GPI-anchored	~1 µg/10^6 cells	(Krantz and Zipursky, 1990)
Drosophila Fasciclin I	glycosylation; GPI-anchored	0.5 mg/liter	(Wang *et al.*, 1993)
Oncogenes			
H-ras (Val^{12} mutant)	Not reported	0.2 – 0.5% of total cellular protein	(Johansen *et al.*, 1989)

Continued on next page

Proteins expressed using *Drosophila* S2 cells, continued

Other proteins expressed using S2 cells

Other cell adhesion proteins (i.e. ARK receptor (Bellosta *et al.*, 1995), fasciclin III (Snow *et al.*, 1989), Toll (Keith and Gay, 1990), neurotactin (Barthalay *et al.*, 1990), and gliolectin (Tiemeyer and Goodman, 1996)) have been functionally tested in S2 cells.

S2 cells are particularly useful for studying transcription factors with no homologues in *Drosophila*. These include the *Arabidopsis* heat shock factor (Hubel *et al.*, 1995), c-Krox (Galera *et al.*, 1994), and SP1 (Courey and Tjian, 1988). Entire *Drosophila* promoters have been dissected in S2 cells (*i.e.* actin 5C (Chung and Keller, 1990), retrotransposon *mdg1* (Arkhipova and Ilyin, 1991), and Doc (Contursi *et al.*, 1995)). Functional analyses have been performed on the insulin-like growth factor I receptor gene promoter (Werner *et al.*, 1992) and defined erythroid promoters (Gregory *et al.*, 1996).

S2 cells have been used as a null background to study homeotic genes and homeodomain-containing proteins such as fushi taruzu, paired, zen, even-skipped, engrailed, ultrabithorax, and antennapedia (Han *et al.*, 1989; Krasnow *et al.*, 1989; Winslow *et al.*, 1989).

Features of the selection vectors

Features of the selection vectors

The table below describes the relevant features of pCoHygro and pCoBlast. All features have been functionally tested.

Feature	Benefit
Drosophila copia promoter	Permits high-level expression of the hygromycin (in pCoHygro) or blasticidin (in pCoBlast) resistance gene
Hygromycin(*HPH*) resistance gene (pCoHygro only)	Allows selection of stable transfectants in *Drosophila* S2 cells (van der Straten *et al.*, 1989)
Blasticidin (*bsd*) resistance gene (pCoBlast only)	Allows selection of stable transfectants in *Drosophila* S2 cells (Kimura *et al.*, 1994)
SV40 early polyadenylation signal	Permits efficient transcription termination and polyadenylation of mRNA
pUC origin	Permits high-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

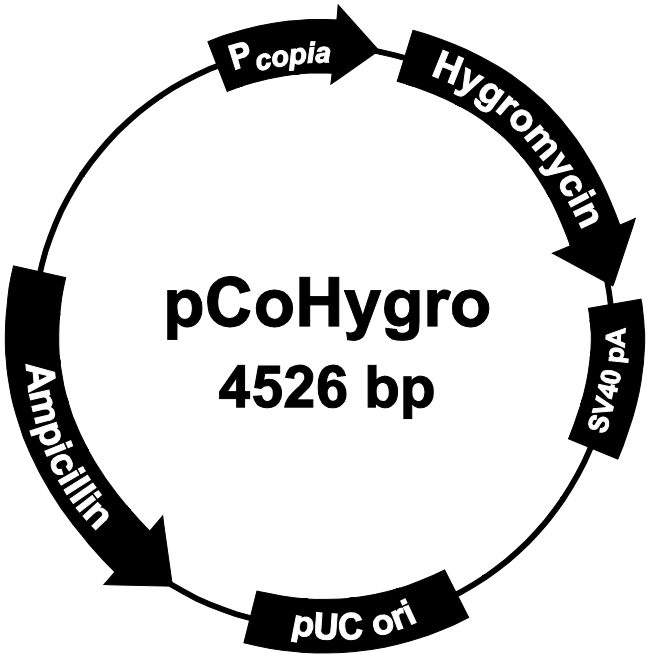
Map of pCoHygro

Description

pCoHygro is a 4526 bp selection vector used to create stable transfectants in *Drosophila* (van der Straten *et al.,* 1989). It contains the *E. coli* hygromycin-B-phosphotransferase gene under control of the *Drosophila copia* promoter for resistance to hygromycin-B in S2 cells.

Map

The figure below summarizes the features of the pCoHygro vector. The nucleotide sequence for pCoHygro is available for download from **www.thermofisher.com** or by contacting Technical Support (page 35).



Comments for pCoHygro
4526 nucleotides

copia promoter: bases 500-746
Hygromycin resistance gene: bases 781-1830
SV40 early polyadenylation sequence: bases 2146-2280
pUC origin: bases 2648-3321 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 3466-4326 (complementary strand)
bla promoter: bases 4327-4425 (complementary strand)

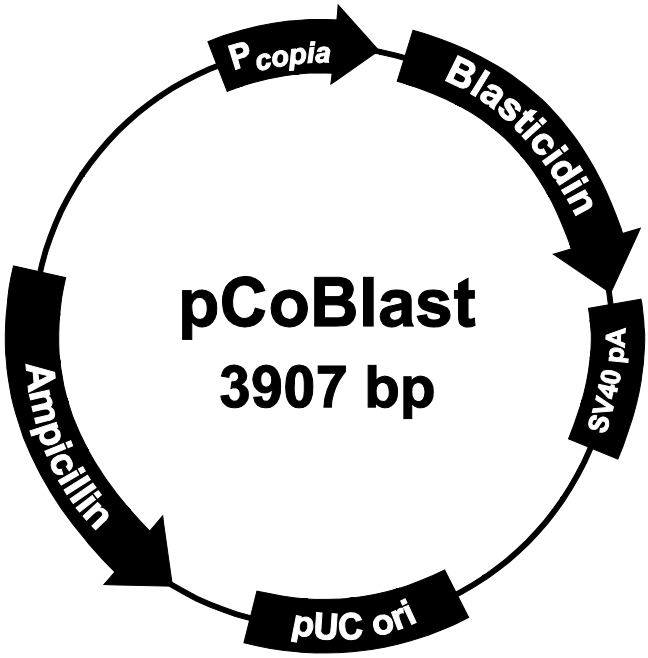
Map of pCoBlast

Description

pCoBlast is a 3907 bp selection vector that can be cotransfected with the expression vector of choice to to create stable cell lines in *Drosophila*. It contains the *Streptomyces griseochromogenes bsd* gene under control of the *Drosophila copia* promoter to confer resistance to blasticidin in S2 cells.

Map

The figure below summarizes the features of the pCoBlast vector. The nucleotide sequence for pCoBlast is available for download from **www.thermofisher.com** or by contacting Technical Support (page 35).



Comments for pCoBlast
3907 nucleotides

copia promoter: bases 500-746
Blasticidin resistance gene: bases 797-1192
SV40 early polyadenylation sequence: bases 1527-1661
pUC origin: bases 2029-2702 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 2847-3707 (complementary strand)
bla promoter: bases 3708-3806 (complementary strand)

Accessory products

Additional products

Many of the reagents supplied in the DES™ kits as well as additional products that may be used with the DES™ kits are available separately. The Blasticidin Support Kit includes pCoBlast, blasticidin, S2 cells, Schneider's *Drosophila* Medium, and transfection reagents. Ordering information is provided below.

Product	Amount	Catalog no.
Schneider (S2) Cells	1×10^7 cells	R690-07
Schneider's *Drosophila* Medium	500 mL	21720-024
Fetal Bovine Serum, heat inactivated	500 mL	10082-147
Penicillin-Streptomycin	100 mL	15070-063
Phosphate-Buffered Saline (PBS), pH 7.4	500 mL	10010-023
PureLink™ HiPure Miniprep Kit	100 preps	K2100-03
PureLink™ HiPure MidiPrep Kit	25 preps	K2100-04
Hygromycin-B	1 gram	R220-05
Blasticidin S HCl	50 mg	R210-01
pMT/BioEase™-DEST Gateway™ Vector	6 µg	V4140-20
pMT/V5-His A, B, and C	20 µg each	V4120-20
pMT/BiP/V5-His A, B, and C	20 µg each	V4130-20
pAc5.1/V5-His A, B, and C	20 µg each	V4110-20
DES™ Blasticidin Support Kit	1 kit	K5150-01
One Shot™ TOP10 Chemically Competent *E. coli*	20 × 50 µL	C4040-03
One Shot™ TOP10 Electrocomp *E. coli*	20 × 50 µL	C4040-52
One Shot™ DH5α™-T1^R Max Efficiency™ Chemically Competent *E. coli*	20 × 50 µL	12297-016
imMedia™ Amp Agar	20 each	Q601-20
β-Gal Assay Kit	1 kit	K1455-01
β-Gal Antiserum	50 µL	R901-25
GFP Antiserum	50 µL	R970-01
Pluronic F-68	100 mL	24040-032

Detecting recombinant protein expressed from pMT/BioEase™-DEST

If you are using pMT/BioEase™-DEST, your recombinant protein will be biotinylated and can be easily detected using streptavidin-horseradish peroxidase (HRP) or streptavidin-alkaline phosphatase (AP) conjugates available for purchase (see table below). The amount of streptavidin conjugate supplied is sufficient for 25 westerns.

Product	Amount	Catalog no.
Streptavidin-HRP Conjugate	50 µL	SA100-01
Streptavidin-AP Conjugate	125 µL	SA100-03

Continued on next page

Detecting recombinant protein expressed from other DES™ vectors

Expression of your recombinant fusion protein can be detected using an antibody to the appropriate epitope. The table below describes the antibodies available for detection of C-terminal fusion proteins expressed using DES™. The amount of antibody supplied is sufficient for 25 westerns.

Product	Amount	Catalog no.
Anti-V5 Antibody	50 µL	R960-25
Anti-V5-HRP Antibody	50 µL	R961-25
Anti-V5-AP Antibody	125 µL	R962-25
Anti-His (C-term) Antibody	50 µL	R930-25
Anti-His(C-term)-HRP Antibody	50 µL	R931-25
Anti-His(C-term)-AP Antibody	125 µL	R932-25

Purifying recombinant protein

Biotinylated proteins expressed from pMT/BioEase™-DEST may be purified by using Streptavidin Agarose, while recombinant proteins expressed from the other DES™ vectors may be purified by using metal-chelating resins including ProBond™ and Ni-NTA. See the table below for ordering information.

Product	Amount	Catalog no.
Streptavidin Agarose	5 mL	SA100-04
EKMax™	250 units	E180-01
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Purification System with Anti-V5-HRP Antibody	1 kit	K854-01
ProBond™ Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K853-01
ProBond™ Nickel-Chelating Resin	50 mL	R801-01
	150 mL	R801-15
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Purification System with Anti-V5-HRP Antibody	1 kit	K954-01
Ni-NTA Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K953-01
Ni-NTA Agarose	10 mL	R901-01
	25 mL	R901-15
	100 mL	R901-10
Polypropylene Columns	50 columns	R640-50

Documentation and support

Obtaining support

Technical support

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  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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