Drosophila
Expression System

Version G

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Drosophila Expression System

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

Catalog nos. K4110-01, K4120-01, K4130-01, K5110-01, K5120-01, K5130-01

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Important Information

Shipping/Storage

Shipping:

- The Schneider's *Drosophila* Medium is shipped at room temperature.
- The rest of the kit is shipped on dry ice.

Storage: Upon receipt—

- Store the cells in liquid nitrogen
- Store the vectors, primers, and Calcium Phosphate Transfection Kit at -20°C
- Store the Schneider's *Drosophila* Medium and copper sulfate (if applicable) at +4°C
- Store the hygromycin-B at +4°C, protected from light (DES® Kits with pCoHygro only)
- Store the blasticidin powder at +4°C (DES® Kits with pCoBlast only)

Kit Contents

The *Drosophila* Expression System (DES®) manual is supplied with the following kits listed below. Each kit contains vectors (specific for each kit), primers, selection reagents, cells, medium, and calcium phosphate transfection reagents (see below and pages v-vi for details).

Twenty micrograms of each lyophilized vector is supplied. Store at -20°C. For information about pCoHygro or pCoBlast, please refer to the **Appendix**, pages 28-30. For information about the expression vector and the control vector, please refer to the specific manual for each vector.

Each kit contains primers for sequencing, but the primers are different depending on the kit. Please see the table below for a list of the primers supplied with each kit. Two micrograms of each lyophilized primer are provided. Store at -20°C.

DES® Kit	Catalog no.	Expression Vector	Control Vector	Primers
Constitutive				
with pCoHygro	K4110-01	pAc5.1/V5-His A, B, and C	pAc5.1/V5-His/*lacZ*	Ac5 Forward
with pCoBlast	K5110-01			BGH Reverse
Inducible				
with pCoHygro	K4120-01	pMT/V5-His A, B, and C	pMT/V5-His/*lacZ*	MT Forward
with pCoBlast	K5120-01			BGH Reverse
Inducible/Secreted				
with pCoHygro	K4130-01	pMT/BiP/V5-His A, B, and C	pMT/BiP/V5-His/GFP	MT Forward
with pCoBlast	K5130-01			BGH Reverse

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Important Information, continued

Primer Sequences

The sequence of each primer is provided below:

Primer	Sequence	pMoles Supplied
Ac5 Forward	5′-ACACAAAGCCGCTCCATCAG-3′	332
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, lyophilized in TE, pH 8	--	20 µg	-20°C
Copper Sulfate*	100 mM	1.5 ml (sufficient for 100 inductions)	+4°C
Hygromycin-B	100 mg/ml	0.5 ml (50 mg)	+4°C, protected from light

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

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, lyophilized in TE, pH 8	--	20 µg	-20°C
Copper Sulfate*	100 mM	1.5 ml (sufficient for 100 inductions)	+4°C
Blasticidin	--	50 mg	+4°C

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

Cells and Medium

- Schneider (S2) Cells: 1 vial, 1 x 10^7 cells/ml in Freezing Medium (see page 5), 1 ml. **Store in liquid nitrogen upon receipt.**
- Schneider's *Drosophila* Medium for S2 insect cells: 2 x 500 ml. Store at +4°C upon receipt.

Invitrogen and Gibco™ Reagents

This product contains both Invitrogen™ and Gibco™ reagents to bring you a high-quality, fully optimized system. You'll get the experience and reliability that Gibco™ brings to cell culture combined with the latest in molecular biology innovation from Invitrogen™.

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Important Information, continued

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/*lacZ* 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 x 12 ml
2X HEPES Buffered Saline (HBS)	50 mM HEPES 1.5 mM Na_2HPO_4 280 mM NaCl pH 7.1	2 x 12 ml
$CaCl_2$	2 M	3 x 1 ml
pcDNA3.1/His/*lacZ*, lyophilized	--	20 µg (for mammalian transfection only)

Reagents Supplied by the User

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

- Heat-inactivated Fetal Bovine Serum (FBS; see page 20)
- Complete Schneider's *Drosophila* Medium (see page 20)
- Penicillin-Streptomycin, if desired (5000 units penicillin G, 5000 µg streptomycin sulfate; Invitrogen, Catalog no. 15070-063)
- 15 ml sterile, conical tubes
- 5, 10, and 25 ml sterile pipettes
- Cryovials
- Hemacytometer and Trypan Blue (see page 20)
- Table-top centrifuge
- 75 cm^2 flasks and 35 mm plates (other flasks and plates may be used)
- Sterile microcentrifuge tubes (1.5 ml)
- Cell Lysis Buffer (see recipe on page 20)
- Phosphate-Buffered Saline (PBS; see recipe on page 20)

Accessory Products

Introduction

The products listed in this section are intended for use with the DES® kits. For more information, please refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 31).

Products Available Separately

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 from Invitrogen. 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 ml vial, 1 x 10^7 cells/ml	R690-07
Schneider's *Drosophila* Medium	500 ml	11720-034
Fetal Bovine Serum	500 ml	16000-044
Penicillin-Streptomycin	100 ml	15070-063
Calcium Phosphate Transfection Kit	75 reactions	K2780-01
Cellfectin® Reagent	1 ml	10362-010
Hygromycin-B	1 gram	R220-05
Blasticidin S HCl	50 mg	R210-01
MT Forward Primer	2 µg, lyophilized	N620-02
BGH Reverse Primer	2 µg, lyophilized	N575-02
Ac5 Forward Primer	2 µg, lyophilized	N621-02
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

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Accessory Products, continued

Detection of Recombinant Proteins

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®. Horseradish peroxidase (HRP) and alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using chemiluminescent or colorimetric detection methods.

Fifty microliters of each antibody is supplied which is sufficient for 25 westerns.

Product	Epitope	Catalog no.
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991) GKPIPNPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection (Lindner *et al.*, 1997) HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25

Purification of Recombinant Protein

The metal binding domain encoded by the polyhistidine tag allows simple, easy purification of your recombinant protein by Immobilized Metal Affinity Chromatography (IMAC) using Invitrogen's ProBond™ Resin (see below). To purify proteins expressed using DES®, the ProBond™ Purification System or the ProBond™ resin in bulk are available separately. See the table below for ordering information.

Product	Quantity	Catalog no.
ProBond™ Purification System (includes six 2 ml precharged, prepacked ProBond™ resin columns and buffers for native and denaturing purification)	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™ Metal-Binding Resin (precharged resin provided as a 50% slurry in 20% ethanol)	50 ml	R801-01
	150 ml	R801-15
Purification Columns (10 ml polypropylene columns)	50	R640-50

Introduction

Overview

Introduction

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 room temperature and do not require CO_2.

The vectors used for expression in S2 cells are very versatile, allowing inducible expression from the metallothionein (MT) promoter (Bunch *et al.*, 1988; Maroni *et al.*, 1986) or constitutive expression from the (actin) Ac5 promoter (Chung and Keller, 1990). Expression can either be intracellular or secreted for simplified purification. Many native signal sequences are functional in S2 cells and can be used to secrete proteins using either the pAc5.1/V5-His or pMT/V5-His vectors. pMT/BiP/V5-His is also available if you want to fuse your protein to a *Drosophila* secretion signal sequence. To facilitate cloning of *Taq*-amplified PCR products, the pMT/V5-His vector is available adapted to topoisomerase I.

In addition, each expression vector encodes a C-terminal peptide containing the V5 epitope for antibody detection and a polyhistidine (6xHis) tag for purification.

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.

Types of DES® Kits Available

Many types of DES® Kits are available from Invitrogen, each containing a different set of expression vectors and your choice of the pCoHygro or pCoBlast selection vector. The DES® TOPO TA Expression Kit is also available for rapid and efficient cloning of PCR products into a topoisomerase-adapted pMT/V5-His-TOPO® vector for inducible expression of heterologous proteins. For more information, please refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 31). Please refer to the table below for a description of each kit.

Kit	Expression Vector	Function	Selection Vector	Catalog no.
DES® Constitutive	pAc5.1/V5-His A, B, and C	Constitutive expression of heterologous proteins	pCoHygro	K4110-01
			pCoBlast	K5110-01
DES® Inducible	pMT/V5-His A, B, and C	Inducible expression of heterologous proteins	pCoHygro	K4120-01
			pCoBlast	K5120-01
DES® Inducible/ Secreted	pMT/BiP/V5-His A, B, and C	Inducible, secreted expression of heterologous proteins	pCoHygro	K4130-01
			pCoBlast	K5130-01
DES® TOPO TA Expression Kit	pMT/V5-His-TOPO®	Inducible expression of heterologous proteins	---	K4125-01

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Overview, continued

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, please refer to the pages indicated.

Step	Action	Page
1	Establish culture of S2 cells from supplied frozen stock.	3-5
2	Develop a cloning strategy to ligate your gene of interest into the desired vector in frame with the C-terminal peptide encoding the V5 epitope and the polyhistidine (6xHis) tag. For diagrams of the multiple cloning site, please refer to the specific manual for each expression vector.	see vector manual
3	Ligate your gene into the desired vector and transform into a *rec*A, *end*A *E. coli* strain (e.g. TOP10). Select on LB plates containing 50 to 100 µg/ml ampicillin.	see vector manual
4	Isolate plasmid DNA and sequence your recombinant expression vector to confirm that your protein is in frame with the C-terminal peptide.	see vector manual
5	Transiently transfect S2 cells.	8-10
6	Induce, if necessary, and assay for expression of your protein.	10-11
7	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.	14-16
8	Induce, if necessary, and assay for expression of your protein	10-11, 17
9	Scale-up expression for purification.	18-19
10	Purify your recombinant protein by chromatography on metal-chelating resin (i.e. ProBond™).	18-19

Methods

Culturing 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 28°C or room temperature without CO_2 as a loose, semi-adherent monolayer in tissue culture flasks and in suspension in spinners and shake flasks.

General Cell Handling

General guidelines are provided below to help you grow S2 cells.

- 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 in a 28°C incubator 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 (Catalog no. 11720-034) and FBS (Catalog no. 16000-044) are available separately from Invitrogen.
- Optional: Use Penicillin-Streptomycin at a final concentration of 50 units penicillin G and 50 µg streptomycin sulfate per milliliter of medium.
- Before starting experiments, be sure to have established frozen S2 cell stocks.
- 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 95-99%.
- Always use **new** flasks or plates when passing cells for general maintenance. During transfection and selection keep cells in the **same** culture vessel.
- For general maintenance of cells, pass S2 cells when cell density is between 6 to 20 x 10^6 cells/ml and split at a 1:2 to 1:5 dilution. **Note**: S2 cells do not grow well when seeded at a density below 5 x 10^5 cells/ml.

  For example, transfer 2 ml of a 10 ml cell suspension at 2.0 x 10^7 cells/ml to a new 75 cm^2 flask containing 10 ml of new medium.
- 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 1000 x g for 2 to 3 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).

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Culturing S2 Cells, continued

Before Starting

Be sure to have the following solutions and supplies available:

- 15 ml sterile, conical tubes
- 5, 10, and 25 ml sterile pipettes
- Cryovials
- Hemacytometer and Trypan blue
- Complete Schneider's *Drosophila* Medium
- Heat-inactivated fetal bovine serum (FBS)
- Optional: Penicillin-Streptomycin (Final concentration: 50 units penicillin G and 50 µg streptomycin sulfate per milliliter of culture)
- Table-top centrifuge
- 25 cm^2 flasks, 75 cm^2 flasks, and 35 mm plates (other flasks and plates may be used)
- Phosphate-Buffered Saline (PBS; see recipe on page 20)

Initiating Cell Culture from Frozen Stock

The following protocol is designed to help you initiate a cell culture from a frozen stock. Note that the vial of S2 cells supplied contains ~1 x 10^7 cells. Upon thawing, cells should have a viability of 60-70%. Once the culture is established, cell viability should be >95%.

1. Remove the vial of cells from liquid nitrogen and thaw quickly at 37°C.
2. Just before the cells are completely thawed, decontaminate the outside of the vial with 70% ethanol and transfer the cells to a 25 cm^2 flask containing 4 ml of room temperature complete Schneider's *Drosophila* Medium.
3. Incubate at 28°C for 30 minutes.
4. Resuspend the cells and centrifuge at 1000 x g for 2-3 minutes. Decant the medium to remove the DMSO and plate the cells in 5 ml fresh complete Schneider's *Drosophila* Medium.
5. Incubate at 28°C until cells reach a density of 6 to 20 x 10^6 cells/ml. This may take 3 to 4 days.

Important

Please note that for the first day after thawing, most of the S2 cells will likely be floating in the medium and will look smaller in size. The majority of these cells are viable although some may take up trypan blue stain. You should consider the following points when working with the cells directly after thawing:

- When removing the medium containing DMSO (see Step 4 above), **do not aspirate** the medium. You will lose all of your cells. Spin the cells down and resuspend in fresh medium as detailed in Step 4 above.
- Do not use trypan blue staining to count the cells. Because some viable cells can take up trypan blue, your cell counts may be inaccurate.

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Culturing S2 Cells, continued

Passaging the S2 Cells

Note: Cells will start to clump at a density of ~5 x 10^6 cells/ml in serum-containing medium. This does not seem to affect growth. Clumps can be broken up during passage.

1. S2 cells should be subcultured to a final density of 2 to 4 x 10^6 cells/ml. Do not split cells below a density of 0.5 x 10^6 cells/ml.

   For example, 2 ml of cells from a 75 cm^2 flask at a density of 2 x10^7 cells/ml should be placed into a **new** 75 cm^2 flask containing 10 ml of fresh complete Schneider's *Drosophila* Medium.
2. When removing cells from the flask, tap the flask several times to dislodge cells that may be attached to the surface of the flask. Use a 5 ml pipette to wash down the surface of the flask with the conditioned medium to remove the remaining adherent S2 cells.
3. Once the cells have detached, briefly pipette the solution up and down to break up clumps of cells.
4. Split cells at a 1:2 to 1:5 dilution into **new** culture vessels. Add complete Schneider's *Drosophila* Medium and incubate at 28°C incubator until the density reaches 6 to 20 x 10^6 cells/ml.
5. Repeat Steps 1-4 as necessary to expand cells for transfection or expression.

Freezing S2 Cells

Before starting, label ~15 cryovials and place on wet ice.

Note: Freezing Medium is 45% conditioned Schneider's *Drosophila* Medium containing 10% heat-inactivated FBS, 45% fresh Schneider's *Drosophila* Medium containing 10% heat-inactivated FBS, and 10% DMSO. **Be sure to reserve medium after centrifuging cells.**

1. When cells are between 1.0-2.0 x 10^7 cells/ml in a 75 cm^2 flask, remove the cells from the flask. There should be 12 ml of cell suspension.
2. Count a sample of cells in a hemacytometer to determine actual cells/ml and the viability (95-99%).
3. Pellet the cells by centrifuging at 1000 x g for 2 to 3 minutes in a table top centrifuge at +4°C. **Reserve the conditioned medium.**
4. Resuspend the cells in 10 ml PBS and pellet at 1000 x g for 2 to 3 minutes.
5. Prepare Freezing Medium (see recipe above) and reserve.
6. Resuspend the cells at a density of 1.1 x 10^7 cells/ml in Freezing Medium.
7. Aliquot 1 ml of the cell suspension per vial.
8. Freeze cells in a control rate freezer to -80°C, or wrap vials in paper towels and place in a well-insulated container lined with more paper towels. Transfer container to -80°C and hold for 24 hours to allow for a slow freezing process.
9. Transfer vials to liquid nitrogen for long term storage.

Important

Optimal recovery of S2 cells requires growth factors in the medium. Be sure to use conditioned medium in the Freezing Medium. In addition, FBS that has not been heat-inactivated will inhibit growth of S2 cells.

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, please refer to the specific vector manual.

General Molecular Biology Techniques

For help with *E. coli* transformations, restriction enzyme analysis, DNA biochemistry, and plasmid preparation, please 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 including TOP10 (Catalog no. C610-00) or DH5α™-T1^R. We recommend that you propagate the pCoHygro and pCoBlast vectors in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A).

For your convenience, TOP10 and DH5α™-T1^R *E. coli* are available as chemically competent or electrocompetent (TOP10 only) cells in a One Shot® format from Invitrogen.

Item	Quantity	Catalog no.
One Shot® TOP10 (chemically competent cells)	21 x 50 µl	C4040-03
One Shot® TOP10 Electrocomp (electrocompetent cells)	21 x 50 µl	C4040-52
One Shot® DH5α™-T1^R Max Efficiency® (chemically competent cells)	21 x 50 µl	12297-016

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.

Maintenance of Plasmids

The pCoHygro and pCoBlast vectors contain the ampicillin gene to allow selection of the plasmids using ampicillin (see pages 28-30 for more information about the vectors).

To propagate and maintain each plasmid, we recommend using the following procedure:

1. Resuspend the vector in 20 µl sterile water to prepare a 1 µg/µl stock solution. Store the stock solution at -20°C.
2. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™-T1^R, JM109, or equivalent.
3. Select transformants on LB agar plates containing 50 to 100 µg/ml ampicillin. For fast and easy microwaveable preparation of Low Salt LB agar containing ampicillin, imMedia™ Amp Agar (Catalog no. Q601-20) is available from Invitrogen. For more information, please call Technical Service (see page 31).
4. Prepare a glycerol stock of each plasmid for long-term storage (see the next page).

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Propagation and Maintenance of Plasmids, continued

Preparing a Glycerol Stock

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

1. Streak the original colony out on an LB plate containing 50 μg/ml ampicillin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 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 14). 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 (see the next page).

Generating the Expression Construct

A separate manual is provided to facilitate cloning your gene of interest into the DES® expression vector. For maps and diagrams of the multiple cloning site, please refer to the specific manual for each vector. The complete sequence for each DES® expression vector is available for downloading from our Web site (www.invitrogen.com) or by calling Technical Service (see page 31). 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 S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) or CsCl gradient centrifugation. The S.N.A.P.™ MidiPrep Kit is a medium-scale plasmid isolation kit that isolates 10-200 µg of plasmid DNA from 10-100 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 type of 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.

DES® Kit	Expression Vector	Control Vector
Constitutive	pAc5.1/V5-His A, B, and C	pAc5.1/V5-His/*lac*Z
Inducible	pMT/V5-His A, B, and C	pMT/V5-His/*lac*Z
Inducible/Secreted	pMT/BiP/V5-His A, B, and C	pMT/BiP/V5-His/GFP

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.

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Transient Transfection of S2 Cells, continued

Before Starting

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

- S2 cells growing in culture (You will need 3 x 10^6 S2 cells per 35 mm plate per transfection)
- 35 mm plates (other flasks or plates can be used, see page 24)
- Complete Schneider's *Drosophila* Medium (see page 20)
- Recombinant plasmid DNA (19 µg per transfection. May be varied for optimum expression.)
- Sterile microcentrifuge tubes (1.5 ml)
- Cell Lysis Buffer (see recipe on page 20)
- Calcium Phosphate Transfection Kit (included in the DES® Kit)

Calcium Phosphate Transfection

For transient transfections, please read the instructions below. 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 x 10^6 S2 cells in a 35 mm plate in 3 ml complete Schneider's *Drosophila* Medium (1 x 10^6 cells/ml).
2. Grow 6 to 16 hours at 28°C until cells reach a density of 2-4 x 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 to 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.
8. Proceed to Step 9, next page.

continued on next page

Calcium Phosphate Transfection, continued

Day 3: Posttransfection

9. Remove calcium phosphate solution and wash the cells twice with complete medium. To wash cells, resuspend cells in complete medium and centrifuge at 1000 x g for 2 to 3 minutes. Decant the medium. Add fresh medium and replate into the same vessel. Continue to incubate at 28°C.
10. **If you are using an inducible expression vector (pMT/V5-His or pMT/BiP/V5-His)**, induce expression when the cells either reach log phase (2-4 x 10^6 cells/ml) or 1 to 4 days after transfection. Add copper sulfate to the medium to a final concentration of 500 µM. For example, to induce a 3 ml culture, add 15 µl of a 100 mM $CuSO_4$ stock. Induce for 24 hours before assaying protein.

Day 4+: Harvesting Cells

11. Harvest the cells 2, 3, 4, and 5 days posttransfection and assay for expression of your gene (see next page). There's no need to add fresh medium or additional inducer.

Note

The secretion signal and the C-terminal tag will increase the size of your protein. Please 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.

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

Detection of Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein by western blot analysis, you may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page viii for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a V5 epitope or a polyhistidine (6xHis) tag. The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, please refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 31).

continued on next page

Transient Transfection of S2 Cells, continued

Testing for Expression

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

1. Prepare an SDS-PAGE gel that will resolve your expected recombinant protein (see below).
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 1000 x g for 2 to 3 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 20 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.
7. Assay the lysate for protein concentration.
8. Mix the lysate with 4X SDS-PAGE sample buffer (e.g. 10 µl of 4X SDS-PAGE with 30 µl of lysate, or equivalent). If you are assaying secreted protein, use 10 µl of the medium and mix with 10 µl of 2X SDS-PAGE sample buffer.
9. Load approximately 3 to 30 µg protein per lane. Amount loaded depends on the amount of your protein produced. Load varying amounts of lysates or medium.
10. Electrophorese your samples, blot, and probe with a suitable antibody (see above).
11. Visualize proteins using your desired method.

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 from Invitrogen. The NuPAGE® Gel System avoids the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen also carries 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, please refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 31).

Assay for β-galactosidase

If you use pAc5.1/V5-His/*lacZ* or pMT/V5-His/*lacZ* as a positive control vector, you may assay for β-galactosidase expression by activity assay using cell-free lysates (Miller, 1972) or by staining the cells for activity. Invitrogen offers the β-Gal Assay Kit (Catalog no. K1455-01) and the β-Gal Staining Kit (Catalog no. K1465-01) for fast and easy detection of β-galactosidase expression.

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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:

- Using 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.

- Using 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.

- Using western blot analysis to assay for GFP protein

  GFP Antiserum is available from Invitrogen (Catalog no. R970-01).

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, please refer to the pMT/BiP/V5-His manual.

continued on next page

Troubleshooting

Cells Growing Too Slowly (Or Not At All).

Cells were split back too far. Do not plate cells at less than 2 x 10^5 cells/ml. Cells will eventually grow back up if they weren't split back too far. If cells do not seem to be growing, replate new 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 S.N.A.P.™ 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.

S2 cells may also be transfected using some lipid-based transfection reagents including Cellfectin® Reagent available from Invitrogen (Catalog no. 10362-010) and dimethyldioctadecylammonium bromide (DDAB) (Han, 1996). For more information about Cellfectin® Reagent, contact Technical Service (see page 31).

Low or No Protein Expression.

- **Gene not cloned in-frame with signal sequence or C-terminal sequence.** 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 C-terminal peptide sequence, expression will not be detected using the antibody to the V5 epitope.
- **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 pMT/V5-His or pMT/BiP/V5-His for inducible expression.

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 of the desired protein 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 available with your 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, please refer to pages 28-30.

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, please refer to the **Appendix**, pages 22 and 23.

Note

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, please note that 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.

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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 to 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 to 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 to 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 20)
- Optional: Penicillin-Streptomycin
- 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 20)
- Calcium Phosphate Transfection Kit (included with the kit)

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Selection of Stable Cell Lines, continued

Stable Transfection Using Calcium Phosphate

Follow the steps below to stably transfect cells. Use a ratio of 19:1 (Expression vector:Selection vector). Set up several transfections with different ratios of expression vector to selection vector to optimize expression of your protein. Include a negative control (empty vector) and a positive control (included with the DES® kit of choice).

Day 1: Preparation

1. Seed 3 x 10^6 S2 cells in a 35 mm plate in 3 ml complete Schneider's *Drosophila* Medium.
2. Grow 6 to 16 hours at 28°C until the cells reach log phase (2-4 x 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)	1 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.
7. Incubate for 16 to 24 hours at 28°C.

Day 3: Posttransfection

8. Remove the calcium phosphate solution and wash the cells twice with complete medium. To wash cells, resuspend cells in complete medium and centrifuge at 1000 x g for 2 to 3 minutes. Decant the medium. Add fresh complete Schneider's *Drosophila* Medium **(no selection agent)** and replate into the same well or plate. Do not split cells.
9. Incubate at 28°C for 2 days.

Day 5: Selection

10. Centrifuge the cells and resuspend in complete Schneider's *Drosophila* Medium containing the appropriate concentration of antibiotic. Replace selective medium every 4 to 5 days until resistant cells start growing out (3 to 4 weeks for hygromycin-B; 2 weeks for blasticidin). Always replate into old plates.

+3 Weeks: Expansion

11. Replate resistant cells into new plates with medium containing the appropriate antibiotic and pass cells at a 1:2 dilution when they reach a density of 6 to 20 x 10^6 cells/ml. This is to remove dead cells. **Note**: Resistant cells may need to be plated into smaller plates or wells to promote cell growth before expansion for large-scale expression or preparation of frozen stocks.
12. Expand resistant cells into 6-well plates to test for expression or into flasks to prepare frozen stocks. Maintain cells in medium containing the appropriate antibiotic.

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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 10 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, please 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).

Important

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 purify your protein. General information for protein purification is provided below. Eventually, you may expand your stable cell line into larger flasks, spinners, or shake flasks to obtain the desired yield of protein. If your protein is secreted, you may want to culture cells in serum-free medium to simplify purification (see below).

Serum-Free Medium

It is possible to maintain hygromycin-resistant or blasticidin-resistant S2 cells in serum-free medium for expression and purification purposes. Please note that addition of hygromycin or blasticidin to some serum-free media can kill even hygromycin-resistant 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 7 days.

If you are culturing cells in serum-free medium and plan to use a metal-chelating resin such as ProBond™ to purify your secreted protein, **please note that adding serum-free medium directly to the column will strip the nickel ions from the resin.** See the information below in **Purification of 6xHis-tagged Proteins from Medium** for a general recommendation to address this issue.

Purifying 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. Please 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 below.

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, please 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 chromotography, you may also dialyze your sample prior to purification on metal-chelating resin. Dialysis will not concentrate your sample.

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Scale-Up and Purification, continued

Metal-chelating Resin

You may use the ProBond™ Purification System (Catalog no. K850-01) or a similar product to purify your 6xHis-tagged protein. The ProBond™ Purification System contains ProBond™, a metal-chelating resin specifically designed to purify 6xHis-tagged proteins. Please refer to the ProBond™ Purification System manual for instructions to purify your protein. If you are using another resin, consult the manufacturer's instructions.

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.

Purification of Intracellularly Expressed Proteins

If you are expressing your 6xHis-tagged protein intracellularly, you may lyse the cells and add the lysate directly to the ProBond™ column. You will need 5 x 10^6 to 1 x 10^7 cells for purification of your protein on a 2 ml ProBond™ column (see ProBond™ Purification System manual).

1. Seed cells at 2 x 10^6 cells/ml in one 25 cm^2 flask.
2. Grow the cells in selective medium until they reach a density of 1 to 2 x 10^7 cell/ml.
3. Harvest the cells by tapping the flask or sloughing the cells.
4. Transfer the cells to a sterile centrifuge tube and centrifuge the cells at 1000 x g for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at -80°C until needed.

Expression of Heterologous Proteins

A number of proteins have been expressed using *Drosophila* S2 cells. A table of these proteins is provided in the **Appendix**, pages 26-27.

Scale-Up

To scale up S2 cell culture, please refer to the table on page 24 for the recommended volumes to use in various culture vessels. On page 25 is a protocol for growing cells in suspension culture in either spinners or shake flasks.

Appendix

Recipes

Trypan Blue Exclusion Assay

1. Prepare a 0.4% stock solution of trypan blue in phosphate buffered saline, pH 7.2-7.3.
2. Mix 0.1 ml of trypan blue solution with 1 ml of cells and examine under a microscope at low magnification.
3. Dead cells will take up trypan blue while live cells will exclude it. Count live cells versus dead cells. Cell viability should be at least 95-99% for healthy log-phase cultures.

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

1X Phosphate-Buffered Saline (PBS)

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4

1. Dissolve: 8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4

   in 800 ml deionized water.
2. Adjust pH to 7.4 with concentrated HCl.
3. Bring the volume to 1 liter. You may wish to autoclave the solution to increase shelf life.

continued on next page

Recipes, continued

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 from Invitrogen (Catalog no. R220-05) in 1 gram aliquots. 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.

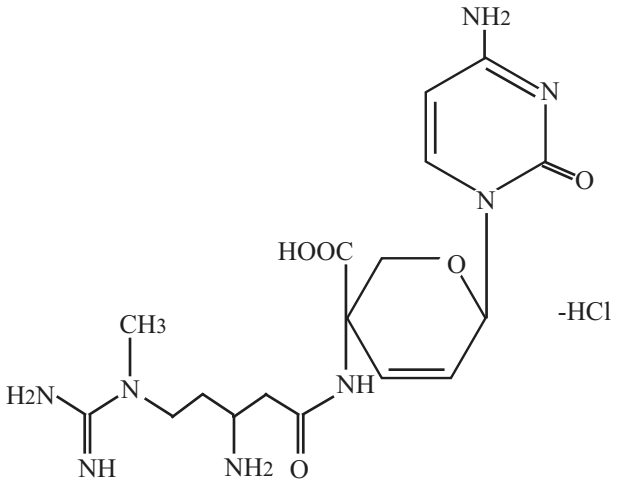
Blasticidin

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

Always 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 from Invitrogen (Catalog no. R210-01) in 50 mg aliquots. 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 (see next to last point below) 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 (1/4 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 (JRH Biosciences, Catalog no. 59-91577P)
- Heparin (Sigma, Catalog 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 (Invitrogen, Catalog no. 24040-032) 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 x 10^6 cells/ml in serum containing medium and >30 x 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, please 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 x 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 (1/4) turn. Increase rate to 140 rpm when cell densities reach >10 x 10^6 cells/ml.
    - Cell viability begins to decrease at densities >12 x 10^6 cells/ml in medium containing serum. Keep cell densities between 5 and 10 x 10^6 cells/ml in serum-containing medium.
    - Cell densities can approach 30 x 10^6 cells/ml without significant decrease in viability in serum-free medium.
4. Subculture cells to ~5 x 10^6 cells/ml when densities reach about ~10 x 10^6 cells/ml.

    Note: If cells are subcultured at densities below 1 x 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 ß-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 anti-body 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)
Receptors			
Human IL5 Receptor α chain (membrane-bound and soluble forms)	membrane protein	17 and 10 mg/liter, respectively 1 x 10^6 sites/cell	(Johanson *et al.*, 1995)

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Proteins Expressed Using *Drosophila* S2 Cells, continued

Table, continued

Protein	Posttranslational Modifications	Expression Level	Reference
Receptors, continued			
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 $\alpha\beta$ 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 $\mu g/10^6$ 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)

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*

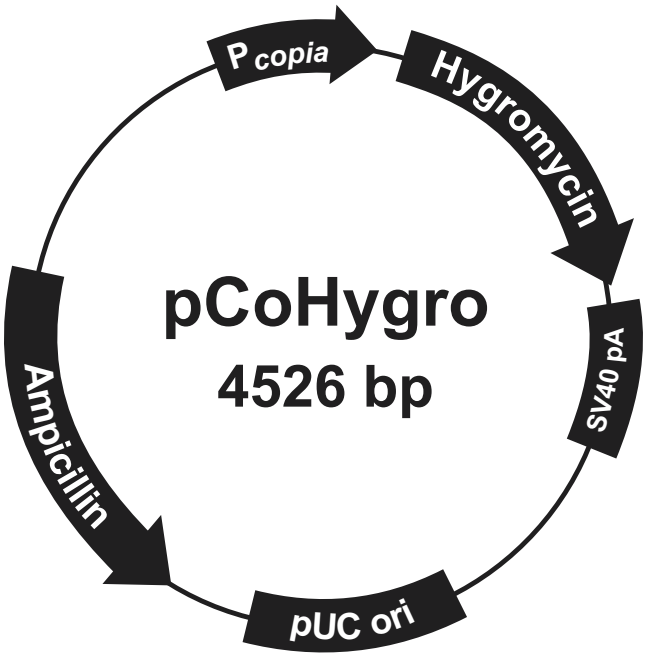
Map of pCoHygro

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 complete nucleotide sequence for pCoHygro is available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service.** See page 31 for more information.



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)

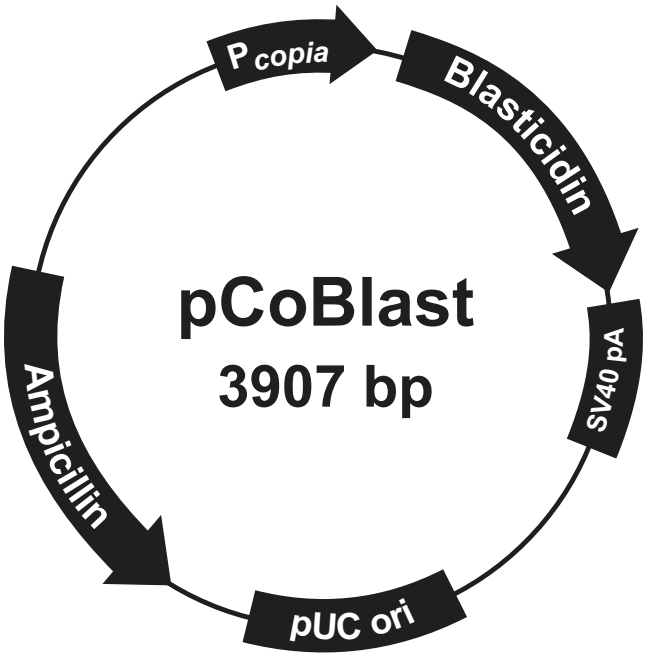
Map of pCoBlast

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 complete nucleotide sequence for pCoBlast is available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service.** See page 31 for more information.



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)

Technical Service

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3. To request additional MSDSs, click the 'Add Another' button.
4. All requests will be faxed unless another method is selected.
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Technical Service, continued

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Japan
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Fax: +81 3-5977-5008

Product Qualification

Introduction

This section describes the criteria used to qualify the components in the DES® kits.

Vectors

Drosophila expression vectors and control vectors are qualified by restriction enzyme digestion. For information about the specific restriction enzymes used to qualify each vector, please refer to the manual for each expression vector. The restriction enzymes used to qualify the pCoHygro and pCoBlast vectors are listed below.

Vector	Restriction Enzyme	Expected Fragments (bp)
pCoHygro	*Eco*R I	1243, 3283
	Nhe I	4526
	Sac II	4526
pCoBlast	*Apa*L I	497, 1246, 2164
	Nsp I	368, 1622, 1917
	Pvu II	753, 896, 2258

Primers

Sequencing primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

Buffers and Solutions

All buffers and solutions are tested for sterility.

Hygromycin B

Hygromycin B is functionally qualified by performing a kill-curve experiment with S2 cells in Schneider's *Drosophila* Medium.

S2 Cells

The following criteria are used to qualify S2 cells:

- Cells are tested independently and certified to be free of mycoplasma.
- Prior to freezing, cells are greater than 95% viable. Forty-eight hours after thawing, cells are greater than 90% viable.

Schneider's *Drosophila* Medium

Each lot of Schneider's *Drosophila* Medium is tested to ensure conformance with the most current approved product specification. This currently consists of tests for pH, osmolality, endotoxin, sterility, and growth of S2 cells. For individual lot test results and more information, contact Gibco™ Technical Service at 1-800-828-6686.

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Product Qualification, continued

Calcium Phosphate Transfection Kit

The components of the kit are tested in a functional assay. A control plasmid in which expression of the chloramphenicol acetyltransferase (CAT) gene is directed by the SV40 promoter is introduced into 293 and CV-1 cells. The level of CAT produced following transfection must be at least 10-fold greater than background (non-transfected 293 cells) and at least 5-fold greater than background (non-transfected CV-1 cells) as determined by ELISA. Transfections are carried out in duplicate and assays are performed in triplicate.

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