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In Vitro Systems

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Basic Protocols for Drosophila S2 Cell Line

Maintenance and Transfection

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Summary

Cells in culture have been increasingly employed in the dissection of intracellular processes. They are generally easier to handle than the organism of study and certainly less complex, which facilitates testing for specific functions and protein–protein interactions. This chapter will describe the extremely simple steps required to keep a healthy S2 cell culture going.

Key Words: Schneider's cells; S2 cells; transient transfections; stable lines.

1. Introduction

In recent years the explosion of interest in and understanding of the molecular underpinnings of the biological clock made it an absolute requirement to possess an alternative system, ideally less complex than the organism under study, to test specific functions or interactions. Usually it is tempting to resort to lower organisms for that task, although the strategy has not always proven successful. In this regard, the clock community has found an ideal venue on the so-called S2 cells, or Schneider's *Drosophila* line 2 cell line. This cell line was established from late (20–24 h) Oregon-R embryos more than 30 yr ago ***(1)***. Originally three independent embryonic lines were established, of which line 2 is the most widespread used. Notwithstanding their somewhat heterogeneous origin the S2 cells are relatively similar in morphology, predominantly epithelial-like in appearance, and range from 5 to 11 µm in diameter and 11 to 35 µm in length. They grow in a loose monolayer with some tendency to remain in suspension. They are mostly diploid, although they have a tendency to become

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tetraploid if seeded too thinly on transfer ***(1)***. According to the American Type Culture Collection, currently they are 60 to 80% tetraploid and they carry exclusively XX chromosomes (*see* **Note 1**).

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This cell line has been used to perform transient expression assays to assess subcellular localization ***(2)***, transcriptional assays ***(3)***, and immunoprecipitations ***(4)***, some of which are described in other chapters in this volume.

2. Materials

1. Schneider's S2 cells. The cells can be obtained from the American Type Culture Collection (www.atcc.org/) or purchased from Invitrogen (www.invitrogen.com, cat. no. R690-07).
2. Schneider's cells medium. This medium can be purchased from a number of vendors; we found the most reasonably priced to be Sigma-Aldrich's (cat. no. S 0146). The composition of the original medium is included in **Table 1**.
3. Fetal calf serum (FCS) or fetal bovine serum (FBS) heat-inactivated at 56°C for 30 min.
4. Antibiotics: penicillin G 50 U/mL, streptomycin sulfate 50 µg/mL.
5. T25, T75, and T150 flasks (Corning).
6. Sterile pipets and technique.
7. Sterile polypropylene tubes (Falcon).
8. Laminar flow hood.
9. Drawer at room temperature (22–25°C) or incubator (28°C).
10. Dimethyl sulfoxide.
11. Freezing medium: Schneider's cells medium supplemented with 20% heat-inactivated FCS and 10% dimethyl sulfoxide.
12. 2-mL Sterile vials.
13. Freezer boxes with foam inside
14. 0.25 *M* $CaCl_2$ filter-sterilized and aliquoted into 15-mL polypropylene tubes at –20°C.
15. 2X HEBES: 16g/L NaCl, 0.7g/L KCl, 0.4g/L Na_2HPO_4, 2g/L dextrose, 10g/L HEPES (as free acid), pH 7.1. After adjusting the pH with NaOH, filter-sterilize and aliquot in polypropylene tubes at –20°C (*see* **Note 2**).
16. 60-mm Petri dishes (Falcon).
17. 17 × 10 mm polycarbonate tubes (Falcon).
18. Selection vectors: pCoHygro or pCoBlast (Invitrogen)
19. Selective drugs: hygromycin or blasticidin (*see* **Note 3**).
20. Lipofectin (Invitrogen) or other lipid-based reagents.
21. Tissue culture-treated Corning (Costar) 6- and 12-well culture clusters.
22. Neubauer chamber.

Table 1

3. Methods

This section outlines how to (1) keep and (2) transiently or stably transfect S2 cells.

Table 1
Composition of Schneider's *Drosophila* Medium

	Molecular weight	Concentration (mg/L)	Molarity (m*M*)
Inorganic Salts			
Calcium chloride ($CaCl_2$)	111	600	5.4
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$	246	3700	15
Potassium chloride (KCl)	75	1600	21
Potassium phosphate (KH_2PO_4)	136	450	2.59
Sodium bicasrbonate ($NaHCO_3$)	84	400	4.76
Sodium chloride (NaCl)	58	2100	35.90
Sodium phosphate, dibasic ($NaHPO_4 \cdot H_2O$)	268	1321	9.57
Other Compounds			
α-ketoglutaric acid	146	200	1.37
D-Glucose	180	2000	11.10
Fumaric acid	116	100	0.862
Malic acid	134	100	0.746
Succinic acid	118	100	0.847
Trehalose	342	2000	5.85
Yeastolate	Nd	2000	Nd
Amino Acids			
β-Alanine	89	500	5.6
L-Alanine	89	400	2.3
L-Aspartic acid	133	400	3.01
L-Cysteine	121	60	0.496
L-Cystine	240	100	0.417
L-Glutamic acid	147	800	5.44
Glycine	75	250	3.33
L-Histidine	155	400	2.58
L-Isoleucine	131	150	1.15
L-Leucine	131	150	1.15
L-Lysine hydrochloride	183	1650	9.02
L-Methlionine	149	800	5.37
L-Phenylalanine	165	150	0.909
L-Proline	115	1700	14.80
L-Serine	105	250	2.38
L-Threonine	119	350	2.94
L-Tryptophan	204	100	0.49
L-Tyrosine	181	500	2.76
L-Valine	117	300	2.65

3.1. Maintenance

1. Seed cells in Schneider's cells medium supplemented with 10% heat-inactivated FCS (or FBS) and antibiotics at 22 to 25°C without gas exchange (*see* **Notes 4** and **5**).
2. Cells are maintained in 25- or 75-cm^2 T-flasks with lids tightly closed. Up to 5 and 10 mL of cells in culture medium can be kept in a T-25 and T-75 flask, respectively.
3. Grow the cells to a density of 1 to 5 × 10^6 cells/mL.
4. Split the culture into fresh medium at a 1:4 or 1:5 dilution every 3 d. Splitting can be pushed to the limit by doing a 1:10 dilution once a week (this for cells kept at 22–23°C; *see* **Note 6**).

S2 cells do not attach well to the plastic surface (or any other solid substrate) and so they are easily resuspended by gently pipetting up and down; alternatively, a rubber policeman can be employed. No trypsinization is required. Doubling time is about 40 h.

For protein expression purposes these cells can be adapted to grow mostly in suspension-employing spinners or shake flasks.

Because S2 cells do not completely adhere to surfaces it is difficult to rinse the cells if needed. To exchange cells into new medium or to wash cells prior to lysis:

1. Resuspend cells in the conditioned medium and centrifuge at 100*g* for 2 to 3 min. Decant the medium.
2. Resuspend the cells in fresh medium (or PBS) and centrifuge as above.
3. Repeat.
4. Add fresh medium (or buffer) and replate the cells (or lyse them).

3.1.1. Freezing and Thawing

As with any other cell line, it is highly recommended to keep track of the number of passages that have taken place since the S2 cells in use were first subcultured (*see* **Note 7**).

To freeze cells down:

1. Grow cells to a density of 3 to 5 ↔ 10^6 cells/mL (log phase) in 30 to 50 mL of medium in a 150-cm^2 T-flask. Alternatively, two T-75 flasks containing approx 15 mL of medium each could be combined into one.
2. Resuspend cells by pipetting with a sterile technique and transfer the medium into a sterile polypropylene tube. Spin in a tabletop centrifuge at 200*g* (about 1000 rpm in an Eppendorf 5810R) for 1 to 2 min.
3. Remove the medium by aspiration and resuspend in 1.5 mL of freezing medium.
4. Aliquot 0.5 mL of cells into 2-mL sterile vials. Label and transfer to a freezer box with foam inside, to allow for slow cooling.

5. Transfer to a –70°C freezer overnight (may be longer).
6. For permanent storage transfer the vials to a liquid nitrogen tank.

To thaw:

1. Remove the vial from liquid nitrogen and warm in a water bath at 25°C (or room temperature).
2. Immediately after the medium is thawed, transfer to a 25-cm^2 (or the equivalent of two vials to a 75-cm^2) T-flask with 5 to 10 mL of Schneider's cells medium with 10% FCS.
3. Allow the cells to loosely attach (about 3 h, but may take longer) and replace the medium with a fresh aliquot.
4. Incubate at 25°C for 3 to 5 d.

After thawing cells may have a long lag period (3 to 7 d) before they start to grow.

3.2. Schneider's Cells Transfection

Drosophila Schneider's cells can be transfected with the expression vector alone for transient expression studies or in combination with a selection vector to create stable cell lines. It is advisable to confirm that there is enough expression of the protein of interest by transient transfection before undertaking selection of stable cell lines. Stable lines are useful for long-term storage, increased expression of the desired protein, and large-scale production. Usually stable cell lines contain several copies of the desired construct, which can be manipulated by varying the ratio of expression and selection plasmids (according to Invitrogen's recommendations; *see* **Note 8**).

Nowadays there are a number of transfection reagents and kits available to transfect this cell line either transiently or stably, the most common ones being from Invitrogen (Lipofectin and Cellfectin), and Qiagen (Effectene). A protocol for transfection with Lipofectin will be described below.

3.2.1. Transfection Assays With $CaCl_2$ Method

AU: Is 17X before the 10-mm tubes in item 3 correct? Unsure about sense.

1. Seed 5 mL of Schneider's cells medium supplemented with 10% FCS (or FBS) and antibiotics in a 60-mm dish with 0.2 to 0.3 mL of cell culture (5 to 8×10^6 cells/mL).
2. Incubate at 25°C for at least 6 h or overnight before transfection.
3. Mix 10 µg of plasmid DNA (expression vector) with 0.4 mL 0.25 *M* $CaCl_2$ and add to 0.4 mL 2X HEBES dropwise, swirling the mix in 17X 10-mm polycarbonate tubes. Incubate at room temperature for 20 to 30 min; the solution should become slightly cloudy.
4. Add 0.8 mL of this solution per 60-mm dish, swirl, and incubate at 25°C (*see* **Note 9**).

To select for stably transformed lines:

1. Repeat the procedure above. However, the plasmid DNA used for transfection is now a combination of expression vector and selection vector (*see* **Note 8**).
2. After 24 h, split cells 1:4 into fresh Schneider's cells medium supplemented with 10% FCS (or FBS) and antibiotics.
3. Wait 24 h longer to add the selective drug.
4. Split cells every 7 to 10 d into fresh Schneider's cells medium supplemented with 10% FCS (or FBS), antibiotics and selective agent (selective medium).
5. Grow cell lines as mixed cultures in selective medium. Eventually the transformed cells should take over the culture.

To clone:

Dilute cells into microtiter plate wells, growing them in a 1:1 ratio of new:conditioned media (sterile-filtered).

3.2.2. Transfection With Lipofectin

Day 1: Plating

1. Under sterile conditions resuspend the S2 cells and proceed to count a 10 µL aliquot in a Neubauer chamber (hemacytometer).
2. Dilute cells to a final concentration of 1×10^6 per mL of fresh Schneider's cells medium supplemented with 10% FCS and antibiotics, seed 0.8 mL per well in a 12-well culture cluster. Cells should derive from a recent subculture (*see* **Note 10**).

Day 2: Transfection

1. Prepare a 1:5 dilution of lipofectin by adding 8 µL of lipofectin per well to 32 µL of Schneider's cells medium per well. Let it sit for 30 to 45 min (*see* **Note 9**).
2. Dilute the recombinant DNA (include the selection vector if stable transfections are sought) in Schneider's cells medium at the proper concentration (*see* **Note 11**). The diluted DNA mix should make up 40 µL per well.
3. Add the diluted lipofectin to the DNA mix. Let it sit for about 10 min.
4. In the meantime, remove the culture media from the wells with a sterile cotton-plugged Pasteur pipet connected to a vacuum device in a laminar flow. Make sure not to remove the loosely attached cells (*see* **Note 12**).
5. Dilute the lipid–DNA complexes up to 400 µL/well in Schneider's cells medium and quickly add dropwise to the side of the wells.
6. Cover with Parafilm. Place in an incubator (or quiet drawer) at room temperature. There is no need to worry about gas exchange.

Day 3: Post-Transfection

1. Add 400 µL of Schneider's cells medium supplemented with 20% FCS.
2. For transient transfections a time course is recommended to determine the optimal harvesting time (*see* **Note 13**).

Special considerations when generating stable cell lines:

1. Wait at least 72 to 96 h after transfection before starting selection.
2. Resuspend the cells, pipetting up and down three or four times. Transfer the cells to a sterile Eppendorf tube and centrifuge at 100 to 200*g* (1000–2000 rpm in an Eppendorf 5415D) for 2 min. Keep the well in the original plate wet by adding 0.5 mL fresh medium.
3. Remove old media and replace with fresh Schneider's cells medium supplemented with 10% FCS and the appropriate selection agent. Add the cells back to the same well.
4. Wrap in Parafilm.
5. Replace selective medium every 4 to 5 d until resistant cells start growing out (generally it takes between 2 and 4 wk depending on the selection agent).

Weeks 2–3: Expansion (Stable Transfection)

1. Wait until the culture reaches a density of 6 to 20 $\times$ 10^7 cells/mL.
2. Centrifuge the cells and resuspend in Schneider's cells medium supplemented with 10% FCS and containing the appropriate selection agent. Passage the cells at a 1:2 dilution plating into smaller plates or wells to promote cell growth.
3. Passage the cells several times before expanding them for large-scale expression or preparing frozen stocks as to remove dead cells.
4. Expand resistant cells into 6-well plates to test for expression or into T-flasks to prepare frozen stocks. Always use the appropriate selection agent when maintaining stable S2 cell lines.

4. Notes

1. Another observation that supports the notion that the Schneider's cells have experienced chromosomal rearrangements along the years in culture is the fact that CLOCK overexpression leads to the induction of the endogenous *timeless* gene (at the mRNA and protein level); meanwhile no expression from the *period* locus (another target of that transcription factor) can be detected (Lino Saez, unpublished observations).
2. When thawing aliquots for use readjust the pH and resterilize right before use.
3. Two common selection vectors are pCoHygro and pCoBlast, both available from Invitrogen. They express the hygromycin or blasticidin resistance genes, respectively, from the *copia* promoter ***(5)***. According to Invitrogen's recommendations, hygromycin is used to a final concentration of 300 µg/mL and blasticidin is used to a final concentration of 25 µg/mL. If using different selection vectors it is advisable to test varying concentrations of the selection agent on the S2 cell line to determine the concentration that kills the cells (kill curve)
4. The S2 cells can be kept in an incubator or even a quiet drawer.
5. Growth can be sped up by culturing at 28°C (not higher).
6. S2 cells grow better when some conditioned medium is brought along with the passage.

7. Cells that have been passaged for an extended time tend to change their growth behavior, morphology, and transfectability. When cells with high passage numbers are used for replicate experiments, decreased transfection efficiencies may be observed in later experiments. We recommend using cells with low passage number (<50 splitting cycles). I have experienced some lack of reproducibility when not taking this matter into account.
8. We used a 19:1 (w/w) ratio of expression vector to selection vector and lipid-based transfection reagents, although the calcium phosphate method is the method of choice in a number of situations.
9. Make sure to include a negative control (empty vector where the recombinant construct was cloned) as well as a positive control (reporter genes such as *luciferase*, green fluorescent protein or *lacZ* are widely used).
10. Ideally the subculture should be 2 to 3 d old but certainly not older than 1 wk.
11. For each new plasmid a titration experiment should be performed. We never used concentrations above 1 µg of plasmid for 10^6 cells (therefore 0.8 µg/well in the 12-well/plate format).
12. It is recommended to transfect no more than four wells at a time to avoid drying out the cells, which will be detrimental to cell viability.
13. When employing lipid-based reagents, transfection efficiency of S2 cells is around 10%. This efficiency can be corroborated employing reporters such as green fluorescent protein or *lacZ* under a constitutive promoter. A number of parameters such as amount of reagent and DNA, length of exposure of cells to the DNA–reagent complex can be optimized for each particular construct. Certain reagents (such as Effectene, Qiagen) provide slightly higher transfection efficiency, although they seem to cause higher degree of cell death (which might not affect certain applications).

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