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Insect cell protocols v2

**Routine maintenance of insect cells**

* The following are comprehensive, but brief, instructions for maintaining insect cell lines. Additional information can be found in the insect cell manual located under the ‘Equipment and Method Manuals’ heading on the Sondek lab website.

1. All insect cell lines are maintained at 27 C with shaking at 140 rpm.
2. Ensure that caps on culture vessels are loosened ~0.5 turn to facilitate aeration.
3. Cell line specific culturing instructions:
   1. Sf9 cells
      1. Maintain cells at a density of 1.0 – 2.0 x 106 cells/mL.
         1. Cells are usually subcultured every other day.
         2. Cells can be split to 0.7 x 106 cells/mL on Friday so that they reach a density of 2.0 x 106 cells/mL on Monday.
      2. Cells are maintained in Grace’s Insect Medium supplemented with 10% Gibco FBS (**not** heat-inactivated), 0.1% Pluronic-F68, and 1X antibiotic-antimycotic.
   2. HiFive cells
      1. Maintain cells at a density of 0.8 – 2.5 x 106 cells/mL.
         1. Cells are usually subcultured every day.
      2. Cells are maintained in ExpressFive SFM supplemented with 10 mM L-glutamine.

General notes for insect cell culture

1. For small scale cultures (≤50 ml), cells are maintained in 125 mL disposable baffled Erlenmeyer flasks.
   1. Cells can be maintained in the same flask until a ring of cell debris has accumulated.
2. For larger cultures (>50 ml), cells are maintained in sterilized glass flasks.
3. Proper cleaning of glass flasks consists of thoroughly scrubbing the flask to remove the ring of cell debris, 3 rinses with tap water, and then 3 rinses with distilled water.
   1. **DO NOT** use detergent or bleach to clean flasks as these chemicals leave a residue that is difficult to completely remove.
4. To ensure sterility, glass flasks must be autoclaved **twice**: once on the liquid cycle (fill flask with distilled water to a level above where the ring of cell debris was), and once on the dry cycle.
5. Recovery of frozen insect cell stocks:
   1. Thaw 1 vial of cells in the 37 C water bath and add to 12 mL of the appropriate culture medium.
      1. Each vial typically contains 1 mL at a density of ~1.0 x 107 cells/mL
   2. Check cell number and viability every day for the first several days, adding medium each day as necessary to achieve a final volume of 50 mL while keeping the cell density at ~1.0 x 106 cells/mL.
   3. Sf9 cells are frozen in 60% Grace’s Insect Medium (without supplements), 30% FBS, and 10% DMSO.
   4. HiFive cells are frozen in 42.5% conditioned ExpressFive SFM, 42.5% fresh ExpressFive SFM, 10% DMSO, and 5% FBS.

**Generating a P0 baculovirus stock**

* The following protocol assumes that fresh bacmid DNA harboring your gene of interest has been prepared. Detailed instructions for cloning your gene into the pFastBac donor plasmid by LIC can be found on the Sondek lab website under the heading ‘Ligation-Independent Cloning Information.’ A protocol for transforming your donor plasmid into DH10Bac *E. coli* can be found in the Bac-to-Bac manual located under the heading ‘Equipment and Method Manuals.’ Instructions for isolating bacmid DNA by mini-prep (AKA the ‘quick and dirty’ protocol) is located under the heading ‘Competent Cell/DNA Protocols.’
* It is advisable to transfect bacmid DNA isolated from several different DH10Bac clones, as clone-to-clone variation in protein expression is very likely.

1. Dilute healthy, log-phase Sf9 cells to 0.5 x 106 cells/mL in supplemented Grace’s Insect Medium and plate 2 mL (1.0 x 106 cells total) into each well of a 6-well dish- allow cells to adhere for 1 hour at 27 C.
2. You’ll need 1 well for each transfection condition.
3. Controls include a mock transfection (cells receive transfection reagent but no bacmid), as well as cells transfected with ‘empty’ bacmid (in other words, bacmid DNA isolated from a blue DH10Bac *E. coli* clone).
4. For each bacmid to be transfected, aliquot 100 μL of SF-900 II SFM into a 1.5 mL micro-centrifuge tube, then add 5 μL of bacmid DNA.
5. **IMPORTANT-** the optimal amount of DNA to transfect must be determined empirically, but 5 μL is usually a reasonable starting point. If you are unsure of how much DNA to use, several different amounts of bacmid can be transfected in parallel.
6. Prepare a stock solution of CellFectin II transfection reagent in SF-900 II SFM.
   1. For each transfection condition, aliquot 10 μL of CellFectin II into 100 μL of media and incubate at room temperature for 3 minutes.
   2. For ease, prepare enough reagent for all transfections in 1 tube.
7. Add 100 μL of CellFectin II/SF-900 II SFM to each tube of diluted bacmid DNA, mix gently by pipette, and incubate at room temperature for 30 minutes.
8. In the meantime, remove the 2 mL of Grace’s Insect Medium from the plated cells and replace with 1 mL of SF-900 II SFM.
   1. This step is necessary to remove all traces of serum, which will lower the transfection efficiency.
9. After the CellFectin II/bacmid DNA complexes have incubated for 30 minutes, add 800 μL of SF-900 II SFM to each tube- the total volume is now ~1 mL.
10. Remove the 1 mL of media on the plated cells and replace with 1 mL of transfection mixture.
    1. Sf9 cells dry out quickly, so it’s best to aspirate the medium immediately before adding the transfection mix.
11. Incubate the cells at 27 C for 5 hours, then aspirate the transfection mixture and replace with 2 mL of supplemented Grace’s Insect Medium.
12. Return plates to the 27 C incubator and harvest the virus-containing supernatant after 5 days- **this is your P0 stock of baculovirus**.
    1. Transfer the supernatant to sterile 1.5 mL tubes and spin at 3,000 rpm for 5 min using the microfuge in the cold room.
    2. Replace 1 mL of ice-cold 1X PBS in each well.
    3. Transfer the cleared supernatant to a new sterile tube and store the virus at 4 C, protected from light.
    4. Scrape up the cells still attached to the plate and combine with any cells that were spun out of the supernatant.
       1. Check for expression of your target protein by SDS-PAGE and Coomassie Brilliant blue staining.
       2. Ensure equal sample loading by measuring the OD600 of each sample prior to resuspending the cells in SDS-PAGE sample buffer.

General notes for generating a P0 stock of baculovirus

1. During the 5 day incubation, plates should be stored in Tupperware lined with moist paper towels to prevent the wells from drying out.
2. The lid of the container should be loose to allow proper aeration and check the towels every day to make sure they are still a little damp.

**Amplifying your P0 baculovirus stock**

* The extent to which the P0 stock of baculovirus needs to be amplified depends on the expression level of your protein. If little or no expression is detected in cells 5 days after transfection with bacmid DNA, you should consider checking the protein expression of a P1 virus. If no expression is detected in P1, further amplification of the virus is not recommended. If protein expression is satisfactory, you can proceed directly to making P2. **Note** that the baculovirus cannot be amplified indefinitely, as the viral genome can acquire deleterious mutations with each round of amplification. Therefore, it’s best to keep an aliquot of P0 virus in reserve for subsequent re-amplification of the virus.

1. Dilute healthy, log-phase Sf9 cells to 0.5 x 106 cells/mL in supplemented Grace’s Insect Medium and plate 2 mL (1.0 x 106 cells total) into each well of a 6-well dish- allow cells to adhere for 1 hour at 27 C.
   1. 1 well is needed for each P0 virus you want to amplify, and be sure to leave an uninfected well on each plate as a control.
2. Add 10 L of P0 virus to each well and swirl the plate gently to mix.
   1. **IMPORTANT-** the optimal amount of virus to add must be determined empirically, but 10 μL is usually a reasonable starting point. If you are unsure of how much virus to use, several different volumes can be added to the cells in parallel.
3. Return plates to the 27 C incubator and harvest the virus-containing supernatant after 5 days- **this is your P1 stock of baculovirus**.
   1. Transfer the supernatant to sterile 1.5 mL tubes and spin at 3,000 rpm for 5 min using the microfuge in the cold room.
   2. Replace 1 mL of ice-cold PBS in each well.
   3. Transfer the cleared supernatant to a new sterile tube and store the virus at 4 C, protected from light.
   4. Scrape up the cells still attached to the plate and combine with any cells that were spun out of the supernatant.
4. Check for expression of your target protein by SDS-PAGE and Coomassie Brilliant blue staining.
5. Ensure equal sample loading by measuring the OD600 of each sample prior to resuspending the cells in SDS-PAGE sample buffer.

**Preparation of a working stock of baculovirus (P2)**

1. Add an appropriate amount of baculovirus to 200 mL of Sf9 cells (density ~2.0 x 106 cells/mL) growing in a 250 mL spinner flask and collect the virus-containing supernatant after 5 days.
   1. The appropriate amount of virus must be determined empirically, however if you are adding P0 virus 1 mL is reasonable starting point. If you are adding P1 virus, add 200-400 L.
2. To harvest the virus, transfer the cell culture to a sterile 200 mL conical centrifuge bottle and spin at 1,200 rpm for 15 minutes at 4 C in the swinging bucket rotor. In the hood, filter the supernatant through a 0.22 m bottle-top filtration device and store the filtered virus at 4 C protected from light.

**Small-scale expression test in HiFive cells**

* Once you have a working stock of baculovirus (P2), you are ready to test the expression of your protein in HiFive cells.

1. Dilute healthy, log-phase HiFive cells to 0.5 x 106 cells/mL in supplemented ExpressFive SFM and plate 2 mL (1.0 x 106 cells total) into each well of a 6-well dish- allow cells to adhere for 1 hour at 27 C.
   1. 1 well is needed for each dilution of virus you want to test, and be sure to leave an uninfected well on each plate as a control.
   2. A range of dilutions from 1:40 to 1:400 is a good place to start.
2. Harvest the cells after 48 hours:
   1. Collect cells that have detached from the plate by spinning the supernatant in a 1.5 mL tube at 3,000 rpm for 5 min using the microfuge in the cold room- aspirate the supernatant.
   2. Replace 1 mL of ice-cold PBS in each well.
   3. Scrape up the cells that are still attached to the plate and combine with the cell pellet from above.
   4. Measure the OD600 of each sample prior to resuspending the cells in SDS-PAGE sample buffer.
   5. Analyze protein expression using SDS-PAGE and Coomassie Brilliant blue staining.

**Large-scale protein expression in HiFive cells**

1. Scale up HiFive cells following the schedule on the tissue culture hood (also included below).
2. When the cell density is ~2.0 x 106 cells/mL, add the appropriate volume of P2 baculovirus as determined by the small-scale expression test above.
3. Harvest the cells after 48 hours:
   1. Collect cells in 500 mL centrifuge bottles and spin at 6,000 rpm at 4 C in the JA-10 rotor for 15 minutes- decant supernatant into a plastic beaker.
   2. Proceed to protein purification, or snap freeze cell pellets in liquid nitrogen and store at -80 C.
   3. Treat supernatant with bleach for 10 minutes prior to disposal in the drain.

**Materials**

GeneMate 125 mL disposable baffled Erlenmeyer flasks (BioExpress F-5909-125B)

Grace’s Insect Medium (Gibco 11605-094)

Gibco FBS (26140-079)

10% Pluronic F-68 (Gibco 24040-032)

100X Antibiotic-Antimycotic (Gibco 15240-062)

ExpressFive SFM (Gibco 10486-025)

200 mM L-glutamine (Gibco 25030-081)

SF-900 II SFM (Gibco 10902-096)

CellFectin II transfection reagent (Invitrogen 10362-100)

Nunc 200 mL conical bottom centrifuge bottle (Fisher 376813)

0.22 m bottle-top filtration system (Corning 431096)

Procedure for expanding HiFive cells

|  |  |  |  |
| --- | --- | --- | --- |
| **day** | **total volume (mL)** | **volume/flask** | **flask** |
| 1 (Monday) | 50 | 50 | 150 mL shaker |
| 2 | 150 | 150 | 2 L |
| 3 | 450 | 450 | 2 L |
| 4 | 1,200 | 300 | 4x 4 L |
| 5 | 4,000 | 1,000 | 4x 4 L |
| 6 (Saturday) | Infect cells with baculovirus | |  |
| 7 |  | |  |
| 8 (Monday) | Harvest cells | |  |

Cells should be split to a density of ~1.0 x 106 cells/mL every day.

Infect cells with baculovirus when the density reaches ~2.0 x 106 cells/mL.