I. Preparing and titering baculovirus working stocks for protein production.

Because we can only send you a small sample of a plaque-purified, first passage (PP1P1) virus stock, you will need to produce and titer a working virus stock before producing your recombinant protein of interest. You should grow the virus by infecting Sf9 or Sf21 cells cultivated in TN-MFH medium containing 10% FCS and 0.1% pluronic F68 ("complete" TN-MFH). Please NOTE that baculovirus stock produced in serum-free media are not stable. For more detailed protocols, see (Jarvis, 2011; Murhammer, 2007; O'Reilly, Miller, and Luckow, 1992), among others.

A) Amplify the PP1P1 stock:

1) Aseptically seed 0.5 X 10^6 Sf9 cells/ml of complete TN-MFH medium into a shake flask.
2) Incubate overnight in a shaking incubator at 28ºC, 125 rpm.
3) Aseptically add ~0.1 mL of the recombinant baculovirus PP1P1 stock.
4) Incubate in a shaking incubator at 28ºC until you see clear signs of infection:
   a) Look for swollen cells, swollen nuclei.
   b) This usually takes ~3-5 days.
5) Aseptically harvest the medium and pellet cells and cell debris:
   a) Ten min at top speed (~1000 g) in a tabletop centrifuge.
6) Aseptically remove the supernatant, transfer to a fresh tube or bottle:
   a) This is the PP1P2 virus stock.
   b) Wrap with aluminum foil to protect from light and store at 4^0C (Jarvis and Garcia, 1994).
      (i) These stocks will slowly lose titer during storage (~1 log/yr; Jarvis and Garcia, 1994)).
      (ii) Can freeze aliquots for longer-term storage/archiving.

B) Titer the working virus stock:

1) Aseptically seed 0.75 X 10^6 Sf9 cells/well into one or more 6-well plates:
   a) Cells will stick best if pelleted, resuspended in serum-free TN-MFH.
   b) Use 3 ml per well for seeding.
   c) Allow cells to adhere for ~1 h at 28ºC.
2) Aseptically prepare a 10-fold dilution series (10^{-1} to 10^{-6}) with PP1P2 stock:
   a) Use complete TNM-FH as the diluent.
   b) Can typically plate (10^{-4}, 10^{-5}, 10^{-6}) and get a useful range of plaques to count.
3) Drain medium from cells.
4) Replace with 2 mL of diluted virus in each well:
   a) Plate each dilution in duplicate.
   b) Incubate for ~1 h at 28ºC.
5) Prepare agarose overlay:
   a) Melt 1.5 mL of 2% agarose/well in a microwave oven.
   b) Equilibrate in a 60ºC water bath.
   c) Warm 1.5 mL of complete 2X Grace's medium/well in an 30ºC water bath.
6) Overlay the infected cell monolayers:
   a) Aseptically mix equal volumes of agarose and complete 2X Grace's medium (3.0 mL/well).
   b) Mix thoroughly.
   c) Aseptically remove the virus from each well in the plaque assay.
   d) Overlay with 3 mL per well.
   e) Allow agarose to harden for 10 min.
7) Seal in a plastic baggie and incubate upside down for 7-10 days at 28ºC.
8) Remove from baggie, count plaques on upside down plate under a dissecting microscope.
   a) Titer = avg # plaques X reciprocal of the dilution (pfu/mL).

II. Protein production and purification.

A) Infect insect cells to produce the protein of interest.

1) Aseptically seed 0.5 X 10^6 Sf9 cells/ml in your favorite insect cell medium into a shake flask:
   a) Best to use Sf9 cells adapted to a serum-free medium to purify a secreted protein.
2) Incubate in the shaking incubator overnight at 28ºC.
3) Perform a viable cell count to determine cell density (should ~double overnight).
4) Aseptically add the volume of virus needed to achieve a multiplicity of infection of 2-5 pfu/cell.
5) Incubate in a shaker-incubator at 28ºC for 2-3 days:
   a) Typically, 48-60 h.
   b) But, best to perform preliminary experiments to determine the time of infection that yields the highest levels of intact product for each recombinant protein.
6) Harvest the medium and pellet cells and cell debris:
   a) Ten min at top speed (~1000 xg) in a tabletop centrifuge.
7) Remove the supernatant, place in a fresh tube or bottle as the cell-free fraction.
   a) For secreted proteins, process the supernatant as the source of recombinant protein.
   b) For intracellular proteins, process the cellular pellet as the source of recombinant protein.
8) For detailed information on immobilized metal affinity chromatography, see:
   a) (Crowe et al., 1994).
   b) (Petty, 2001).
   c) Vendors instructions.
   d) Many papers on HIS-tagged proteins expressed in baculovirus-infected insect cells.

B) Metal affinity-purify the protein of interest (secreted proteins).

1) Dialyze the supernatant against dialysis buffer (50 mM NaH_2PO_4, 500 mM NaCl; pH 8.0) for 12-18 h with a buffer change after 6 h.
2) Wash Probond (Invitrogen) or your favorite metal affinity resin with dialysis buffer.
3) Incubate the dialyzed supernatant with the washed resin at RT for 3 h with gentle agitation.
4) Transfer into an empty column barrel and allow the resin to settle into a bed.
5) Wash with 5 column volumes of dialysis buffer containing low concentration of imidazole:
   a) We use 20-50 mM imidazole for washes.
6) Elute with 1 column volume of dialysis buffer containing high concentration of imidazole:
   a) We use 250 mM imidazole.
7) Assess purification by analysis of starting material, unbound, wash, and elution fractions.

C) Metal affinity-purify the protein of interest (intracellular proteins).

1) Wash the infected cells once or twice with ice cold TBS (50 mM Tris, 100 mM NaCl; pH 8.0).
2) Lyse the cells in one of two ways:
   a) Resuspend pellet in a minimal volume of ice-cold dialysis buffer; freeze-thaw twice.
   b) Resuspend pellet in a minimal volume of TBS + 1% NP40); incubate for 10 min on ice.
3) Shear DNA by tituration with an 18-gauge needle until the solution is no longer viscous.
4) Centrifuge for 15 min at ~10,000 xg at 4ºC.
5) Harvest the supernatant and use for metal affinity chromatography as described above (D2).

III. References.

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