John Zinder's revised, cobbled-together Bac-to-Bac protocol:

Starting from pFastbac/pLib/pBig plasmids or similar (must contain genes of interest with associated promotors and terminators between the Tn7L and Tn7R elements). For pBig cloning see Weissmann F et al., *PNAS* **2016**.

Hybrid of Life Technologies' protocol and David Barford's (Zhang et al., *Methods* **2016**).

Bacmid generation (3 days)

Day 1:

Morning

-Transform 10-100 ng plasmid (volume <1 µL) into 10-20 µL DH10Bac (Thermo cat 10361012) cells by standard heat-shock protocol (45 sec at 42^{o}C).

-Rescue with 100-200 µL SOC or LB media, and incubate at 37^{o}C with shaking for 3-4 hrs.

NOTE: We buy fresh comp cells from manufacturer. I have not had great luck with making my own competent DH10Bac cells despite doing this for nearly every other kind of E. coli. I usually transform 5-10 different plasmids in parallel for this reason. If you must freeze away extra cells do so with isopropanol/dry ice bath and not liquid nitrogen.

Afternoon

-Plate 50-100 µL cells onto LB-agar containing 50 µg/mL kan, 7 µg/mL gentamycin, 5 µg/mL tetracycline, 100 µg/mL Xgal, 40 µg/mL IPTG (~170 µM). I typically plate 50 µL of up to 3 clones per plate, as these plates are a pain to make. Store plates in the dark (tetracycline is light-sensitive).

-Grow at 37^{o}C for at least 20 hr.

Day2:

Afternoon or evening

-Pick two white colonies per clone from the plates and inoculate each into 4 mL LB containing 50 µg/mL kan, 7 µg/mL gentamycin, 5 µg/mL tetracycline. Grow overnight at 37^{o}C with shaking.

-Optional: spot 10 µL of these liquid cultures onto LB-agar/Kan/Gent/Tet/Xgal/IPTG plates and grow for 48 hrs to ensure that they were truly white colonies (do this with a blue colony as well to make sure the plate is still good).

NOTE: I pick two colonies rather than one per clone so I have a backup in case one clone doesn't grow well or has poor yields of bacmid after purification.

Day3:

-Optional (but I always do this): Make a glycerol stock by adding 1 mL of culture to 500 µL 60% glycerol, mixing, flash freezing in liquid nitrogen and storing at -80^{o}C.

-Centrifuge, decant sup, and resuspend pellet in 300 µL 15 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0, 100 µg/mL RNase A (store this solution at 4^{o}C).

-Add 300 µL room temp 200 mM NaOH, 1% w/v SDS and mix well.

-Add 300 µL cold 3 M KOAc pH 5.5 to this solution and mix well.

-Transfer to an Eppi and spin at 16,000-20,000 x g for 10 min to pellet the potassium dodecyl sulfate and chromosomal DNA.

-Transfer the supernatant into a 2 mL tube containing 1 mL isopropanol.

-Spin at 16,000 x g for 10 min at 4^{o}C.

-Decant the supernatant and wash the pellet with 1 mL of 70% v/v ethanol, then spin again.

-Use the vacuum at your bench to decant as much of this supernatant as possible (careful not to suck up the pellet!) and air-dry tubes for 30-120 min.

-Resuspend the pellet (bacmid plus some chromosomal DNA) in 50 µL sterile TE buffer (10 mM Tris8, 1 mM EDTA) and measure A_{260}. Concentration should be in the range of 500-3000 ng/µL.

-Store the bacmid away from light at 4^{o}C and use within a month.

Baculovirus generation (~11 days):
-This assumes you are maintaining a suspension culture of Sf9 cells (Thermo 12659017 or similar) in SF900 III or SF900 II media (Thermo 12658027 or similar) at between 8e5 and 8e6 cells/mL and >90% viability throughout. Also culture Tnis (Expression systems 94-002S) in suspension in ESF921 media (Expression systems 96-001-01) at between 8e5 and 8e6 cells/mL and >90% viability throughout. Viability can be determined with tryptan blue dye and a hemocytometer.
-Tnis have a tendency to clump, making them difficult to accurately count. This is especially evident after thawing DMSO stocks. To help with this, after thawing I spin the cells down and resuspend in fresh media. Then after 2-3 days of spinning in the incubator, I pour the culture into a 50 mL conical, wait 2-3 min for the clumps to fall to the bottom, then transfer the supernatant back into a fresh spinner.
-Suspension Sf9s can also be used for large-scale protein expression but I have had much better luck getting high expression levels with Tni cells.
-1x Pen/Strep and Fungizone (amphotericin B) can be added to the media if needed. I usually add Pen/Strep (except for during transfection) but have only needed to use Fungizone for adherent and not suspension cultures.
-I fill some trays with MQH_2O to keep the 27^{o}C incubator humidity above 65%.
-Note that infected Sf9s will pretty much stop dividing after day 1 of infection. Look at them under the microscope at the beginning and after 2-3 days of infection to confirm this.
-Maintain proper sterile technique throughout. I usually work in the TC hoods when handling cells and media.
-The protocol can be paused at any point after harvesting the virus (e.g. the P1 doesn't need to be immediately used for P2 amplification etc.).
-The media should be stored in the cold room and warmed up to room temp or 27^{o}C before use (but **NOT 37^{o}C**).

<u>Day1</u> (Transfection and P1)
-Plate 3e6 Sf9 cells in 2 mL of SF900 II media in each well of a 6-well plate. Do two wells for each bacmid, so you can store some extra P1 in the end.
-Place in the 27^{o}C incubator for >15 min, during which time they will adhere to the surface.
-During this time, transfer your bacmids into the cell culture hood.
-Add 10 µL Cellfectin II (Thermo 10362100) to an Eppi containing 200 µL antibiotic-free SF900 II media.
NOTE: Though I've never confirmed, Pen/Strep and Fungizone are cytotoxic when the cells are being transfected and should be avoided.
-Add 20 µL bacmid to this and mix.
-Incubate bacmid/cellfectin mixtures in the hood for 20-30 min.
-Bring the cells back into the hood and aspirate their media.
-Add 800 µL antibiotic-free media to the tubes containing the bacmid and Cellfectin II and add all ~1 mL of this dropwise to the cells.
-Incubate in the 27^{o}C incubator for 3-5 hr. I usually do 4 hr.
-Make a diluted media mix by adding 5 mL ddH_2O to 25 mL SF900 II/PenStrep/Fungizone (to account for evaporation).
-Aspirate the transfection mixture
-Add 3 mL of this diluted media mix to each well
-Place in the 27^{o}C incubator.

96 hrs later
-Transfer the media (pool like samples) to sterile 15 mL conicals containing 500 µL bovine calf serum (or FBS if you're HHMI funded). Spin at 1000 x g for 5 min and transfer the supernatant into labeled Eppis. This is the P1 viral stock.

NOTE: At this point I use 1 mL for the P2 amplification, store 1 mL in the dark at 4°C, and flash freeze the rest and store at -80°C. At 4°C, the stock should be stable for months and at -80°C it should be stable for years.
NOTE: BCS/FBS are added as a cryoprotectant and a substrate for any proteases that may be present in the media (either secreted or from lysed cells).

Day 5 (starting P2 viral amplification):
-Plate 30e6 Sf9 cells in a total of 30 mL 5:1 SF900 II : sterile H_2O on a 15 cm dish.
-Place in the incubator for 15-30 min. Cells should fully adhere to the dish during this time.
-Bring the plate back into the hood and add 1 mL of P1 to it dropwise, covering the whole plate, then mix by gently rocking the plate back and forth.
-Place the plate back in the 27°C incubator.

Day 8 (P2 harvest and P3 amplification)
-Plate 2 x 30e6 Sf9 cells (per baculovirus clone) in a total of 30 mL 5:1 SF900 II : sterile H_2O on a 15 cm dish.
-Place in the incubator for 15-30 min. Cells should fully adhere during this time.
-Harvest the P2 by decanting the media into a 50 mL conical containing 5 mL BCS or FBS, spinning at 1000 x g for 5 min, then decanting that into two labeled 15 mL conicals.
-Store half in the dark at 4°C and flash-freeze the rest for storage at -80°C.
-OPTIONAL (preliminary expression test): instead of decanting the media prior to spinning, scrape the cells and transfer them into a 50 mL conical. After decanting and storing the supernatant, resuspend the pellet in 5 mL cold PBS. Pellet 100 µL of this in an Eppi and resuspend in 100-200 µL 1x Laemmli buffer + BME. Boil for 5 min, then use the bath sonicator in the cold room (high setting, 5 min) to break up the cellular DNA. Load 5-10 µL of this for SDS PAGE/Coomassie blue staining and/or Western analysis.
-Bring the plates back into the hood and add 1 mL P2 stock to each of them as before.
-Place the plate back in the 27°C incubator.
NOTE: If you need to scale up, start more than 2 plates for your P3.

Day 9 (if doing suspension expression in Tnis)
-Dilute Tni suspension cells to a final concentration of 4e5 cells/mL in a volume of 400 mL ESF921 in a 1 L spinner. Grow at 27°C with spinning. They should reach ~2e6 cells/mL by the time you're ready to infect them.

Day 11 (Harvest P3):
-Harvest the P3 and use it immediately. No need to spin as the floating Sf9 cells will also secrete virus and increase expression. I have not had much luck storing P3 stocks.

Protein production (72 hrs)
500 mL Suspension:
Day1
-Add all of your P3 volume to the Tnis you started on Day 9 of the viral amplification and incubate at 27°C with stirring for 3 days.
-Optional: Take a 0 time point by resuspending 5e5 cells in 200 µL 1x Laemmli buffer + BME, boiling and sonicating.

Day 4:
-Spin cells down at 500 x g for 5 min and discard the supernatant (into bleach!).
-Using a metal spatula, transfer the cells into a blocked 10 or 20 mL syringe.
-carefully insert the plunger until it snaps, invert the syringe, then remove the block once the cells have moved from the outlet.

-Carefully syringe the cells into conicals filled with liquid nitrogen. They should form nice pellets.
-Store at -80^{o}C. These pellets should last ~indefinitely.
-Optional: Cryo-grind pellets to increase extractions efficiency.
NOTE: You can take time points if you'd like by spinning down aliquots after each day and preparing an SDS-PAGE sample as previously. Don't expect any protein expression after 1 day though, as the polyhedron promoter takes ~24 hr to start expression.

Adherent culture in 15 cm plates:
Day1:
-Plate 30e6 Sf9 cells in a total of 30 mL SF900-II/PenStrep/2.5% FBS(optional)/17% water on as many 15 cm dishes as you'd like.
-Place in the incubator for 15-30 min.
-Add 1 mL of the P3 stock to each plate.
-Place plates back in the incubator.

Day 4:
-Scrape cells and pool into 50 mL conicals.
-Spin cells down at 100 x g for 5 min in 50 mL conicals and discard the supernatant. I typically do this 2 times for each conical, so that the resulting pellet is derived from ~100 mL of culture. Note that the volume will be decreased due to evaporation.
-Decant as much of the supernatant as possible after the last spin and flash freeze the pellet in liquid nitrogen.
-Store at -80^{o}C. These pellets should last ~indefinitely.

General notes
-I greatly prefer Tni suspension culture for protein production but you gotta do what you gotta do. Some proteins may only express in adherent Sf9s.
-Many people titer their viruses and calculate multiplicity of infection etc. to determine how much to add for expression and amplification steps. While this is a very good idea it is time consuming and I do not do it. This protocol aims to saturate each amplification by adding an extra day per step, and so it's relatively predictable how strong your P3 will be and thus what volume you'll need for your protein production infection.
-The P2 can be used for protein expression because it should be approx the same titer as the P3. The only reason for doing the P3 amplification is so you can scale up (I.E. 10 mL of P2 becomes 250 mL of P3). Sometimes if I want to get protein 3 days earlier, I'll double my P2 amplification and use half of it as if it were a P3 for infection.