BMC	**Standard Operation Procedure** **Preparing a selenomethionyl-labeled protein in BV-Sf9 insect cells**

1. **Aim:**

   Heavy atom (Selenomethionine)-labeling of proteins produced in baculovirus-assisted expression in Sf9 insect cells

2. **Applicability:**

   Selenium can be used for phase determination in multi-wavelength anomalous diffraction (MAD) method. Se-Met can often replace methionine residues in a protein without affecting the protein's properties, therefore producing a protein advantageous for crystal structure solving. Also, the X-ray absorption edge of selenium is easily accessible by synchrotron radiation, making a Se-Met crystal ideal for collecting anomalous X-ray diffraction data.

3. **Responsibilities:**

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4. **Required materials:**

   WARNING! SeMet is TOXIC. The culture supernatant should be collected for disposal as hazardous waste.

   Antibiotics (Calbiochem, DE)

   ESF 921 Insec Cell Culture Media, Protein-free and Without Methionine (Expression system, USA)

   L(+)-Selenomethionine, >99% (Acros Organics, USA)

   Ni-NTA resin (Merck, Germany)

   Gentamicin solution G1397 100 ml (Sigma-Aldrich, USA)

   β-mercaptoethanol (βME) (Sigma-Aldrich, USA)

   PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific, MA, USA)

   Vivaspin® concentrators (Sigma-Aldrich, USA)

   Sf9 insect cell line CRL-171TM (ATCC, UK)

   Insect-XPRESS TM medium w/L-Gln, (protein-free insect cell medium with L-Glutamine) (Lonza)

   EZ Blue Gel Staining Reagent (Sigma-Aldrich, USA)

   Amicon Ultra (Merck Millipore, USA)

   Mini-PROTEAN® Tetra Cell apparatus (Bio-Rad, CA, USA)

5. Procedure:

A. Preparation of high titer virus stock (amplification of recombinant bacmids)

Day 1-6

Seed 14 × 10^6 Insect XPRESS media adapted cells in each T-175 flask in final volume of 50 ml Insect XPRESS protein-free media. Infect cells with 500 µL (~8-10 drops) of low titer recombinant virus stock (prepared earlier, stored at 4 °C) in each T-175 flask to MOI = 1.0. Incubate the cells at 28 °C for 6 days. Harvest the supernatant from the flasks via centrifugation at 1000 g for 10 min. Store the virus supernatant (recombinant bacmids) in a sterile bottle at 4 °C in the dark. It is recommended to use this within 1-3 days.

B. Infection of Sf9 cells with recombinant bacmids in methionine-rich media

Day 7-8

Seed several Erlenmeyer 2L-flasks with healthy dividing insect cells at high viability (> 98%) and at densities of 2 – 4 × 10^6 cells/mL. Infect seeded cells at MOI = 3 – 5 with prepared HTVS solution containing recombinant bacmids harvested on Day 6. Usually it is between 50-100 mL/1L cell culture. Add fresh ESF-921 protein-free media to total volume of 1L per flask. Incubate the flasks at 28 °C for 8 - 36 hours. Most of the proteins required minimum of 16 hrs.

Note: When doing it for the first time, consider using time step analysis. Collect the sample for WB or SDS-PAGE at each 8 hrs of protein production.

C. Depletion of methionine from intracellular pools

Day 9: Washing

Gently spin down the cells at 300 g for 15 min at RT. Collect the sample of supernatant for test on WB or SDS-PAGE. Resuspend the cells in an equal volume of ESF-921 methionine-free media with antibiotics (50 µg/mL gentamycin). This has to be added immediately after resuspension (1 mL of gentamycin solution 50 mg/ml = 50 mg per L).

Note: While resuspending pellet from one flask, the other flasks should be shaking to prevent cells from dying.

Day 10: Depletion

Grow cells for 4-12 hours to deplete intracellular pools of methionine. Gently spin down the cells at 300 g for 10 min at RT. Resuspend the cells in an equal volume of ESF-921 methionine-free media. Add 50mg/L SeMet.

Tip: Weighted powder in eppi and then powdered into flask directly.

Note: The critical point of SeMet addition is within the first 16-20 hours following viral infection, since the addition of SeMet at 24 hours post-infection is too late as protein-label-free starts expressing.

Day 11-14: Expression

Grow cells for next 48 - 96 hours post-infection at 28 °C. Usually, it is enough to do so for 3 day. Harvest the protein that is secreted in the supernatant from the 2000 ml Erlenmeyer flasks by centrifuging at 1000 g for 10 min. Spin supernatant at 5500 g for 10 min and proceed with affinity and gel filtration purification as for native protein.

D. Finaly purified Se-Met-labeled protein

Day 15

In the final step of purification process, the protein incorporated with selenomethionine was loaded onto Superdex 200 column with an internal diameter of 20 mm and length of 30 cm connected to AKTA Pure system (GE Healthcare). Gel filtration was performed using HEPES (pH 7.5) at a flow rate of 0.5 mL/min. The protein was eluted and the process was monitored by measuring UV absorbance at 280 nm. The collected fractions (0.5 mL) were analyzed by SDS-PAGE and analytical SEC. The selected fractions were concentrated, and the final protein concentration was determined using NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The yield of purified protein labeled with selenomethione obtained from isolation from BV-mediated Sf9 insect cells was usually between 0.5 mg/L – 1.2 mg/L.

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