BMC	Standard Operation Procedure Refolding by dialysis from insoluble fraction of bead mill-lysed Ecoli cells

1. **Aim:**

   Efficient refolding of Escherichia coli-expressed signaling receptor

2. **Applicability:**

   All biological molecules with problematic refolding action after bacterial expression

3. **Responsibilities:**

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4. **Required materials:**

   Tryptone, yeast extract, and agar (Difco, USA)

   E. coli BL21(DE3) competent cells (ABO, Poland)

   Plasmid Mini Kit (Promega, USA)

   Ni- NTA resin (Merck, Germany)

   Ball mill (PM100; RETSCH GmbH, Germany)

   Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, USA)

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

   BlueStar PLUS and BlueEasy Prestained Protein Ladders (ABO, Poland)

   Vivaspin® 20 concentrators (Sigma-Aldrich, USA)

   Expression plasmid (Gene of interest in pET-15b) (GenScript, China).

5. **Procedure:**

   A. Protein expression and isolation from bead mill-lysed E.coli cells

   Day 1-2

   The expression plasmid was used for the transformation of E. coli BL21(DE3) competent cells. The culture was grown at 37 °C in LB medium supplemented with 100 µg/mL of ampicillin until the optical density reached a value of 0.6 at a wavelength of 600 nm (OD600). The culture was cooled to 20 °C, and then induction with the final concentration of 0.5 mM of IPTG was carried out.

   Culture samples were withdrawn after 2 h, 4 h, and 16 h and analyzed using sodium SDS-PAGE and Western blot analysis. The expression level reach its maximum usually after 16 h of incubation.

The cells were pelleted by centrifugation at 6000 g for 10 min (Beckman) and frozen as noodles in liquid nitrogen.

Day 3

Frozen E. coli cells (25g) were crushed using ball mill, with a 125 mL grinding stainless steel jar and 7–11 grinding balls (20 mm) of the same material. Jar and balls were prechilled with liquid nitrogen.

The cells were ground for at least four cycles, with each cycle lasting for 3 min, including reverse rotation after 1.5 min and submerging the closed jar in liquid nitrogen between the cycles. Grinding speed was set at 220 rpm for the first cycle and 300 rpm for the subsequent cycles. The cells were suspended in 100 mL of binding buffer (20 mM Tris-HCl (pH 7.9), 150 mM NaCl, and 20% glycerol). After 1 h of incubation on ice, the sample was centrifuged at 16,000 g for 30 min at 4 °C. The supernatant was transferred to a tube and the pellet was resuspended in 100 mL of binding buffer and incubated for 30 min. Then, the suspension was centrifuged at 4 °C and both obtained supernatants were analyzed by SDS-PAGE and Western blot analysis. The supernatants were used for the purification from the soluble fraction, however the rest proceeded for the recovery procedure.

B. Recovery from the insoluble fraction by dialysis

Day 4-5: Solubilization

The pellet derived following cell lysis and centrifugation of the cell suspension without denaturant was further suspended in 100 mL of binding buffer containing urea (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole, 6 M urea, and 10 mM βME). After overnight incubation with continuous stirring, the suspension was centrifuged at 16,000 g for 30 min at 4°C. The supernatant was transferred to a tube and the pellet was resuspended in 100 mL of binding buffer (with urea), and after incubation for a short period of time, the suspension was centrifuged at 16,000 g. Both supernatants were analyzed by using SDS-PAGE and Western blot techniques.

To use efficient method that facilitates in vitro protein renaturation, the supernatant obtained from the previous step was purify using IMAC and refolded using dialysis. The final step of the purification process was performed by gel filtration technique.

Day 6: Afinity purification

The supernatant, after solubilization in binding buffer with urea, was applied onto the column with Ni-NTA resin and flow-through fractions were applied twice. The resin was washed with 20 CVs of binding buffer containing urea (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole, 6 M urea, and 10 mM βME) and with 10 CVs of each wash buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 20 mM imidazole, and 10 mM βME) containing descending concentrations of urea (6 M, 4 M, 2 M, 1 M, and 0.5 M). Gravity elution was performed with 3 CVs of elution buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 1 M imidazole, and 0.5 M urea), and 2-mL fractions were collected. The elution step was repeated twice. Fractions from the first and second elution cycles were analyzed by SDS-PAGE electrophoresis.

Day 7-9: Volume reduction and refolding

The samples showing the presence of protein of interest were selected and combined, their volume was reduced by centrifugal concentrators, and then the resultant solution was dialyzed at 4 °C against elution buffer without urea.

Afterward, the protein was subjected to dialysis at 4 °C against the refolding buffer (10 mM Tris-HCl pH 7.9, 0.2 M arginine, 1 mM EDTA, 20% glycerol, 2 mM GSH, and 1 mM GSSG). After exposing the sample to refolding conditions for 60 h, the protein solution was concentrated to 250 µL.

C. Gel filtration of refolded protein extracted from the insoluble fraction

Day 10

In the final step of purification process, the protein refolded by dialysis was loaded onto Superdex 75 column with an internal diameter of 10 mm and length of 30 cm connected to AKTA system (GE Healthcare). Gel filtration was performed using PBS (pH 7.4) 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 obtained from the insoluble fraction was usually between 1.2 mg/L – 1.8 mg/L when the refolding procedure was performed by dialysis.

Note: Renaturation is a critical step in the recovery process of any biologically active protein from an insoluble fraction. There are several approaches to protein refolding that need to be considered. Refolding can be initiated by reducing or removing the chaotropic solvent. In addition, parameters such as pH, redox potential, buffer additives, and protein concentration need to be empirically optimized for each individual protein. The best strategy is to screen different refolding conditions in parallel to find the appropriate combination that results in the highest recovery of active protein and shows least amount of soluble multimers.

The development of this SOP was supported by the programme Interreg V – A Slovakia - Austria, project CAPSID.