BMC	Standard Operation Procedure On-column refolding from Ecoli inclusion bodies

1. **Aim:**

   On-column refolding of an antigen (or protein fragment) isolated from inclusion bodies by using a glass dounce homogenizer for extraction

2. **Applicability:**

   For biomedicaly relevant molecules with problematic refolding and/or extraction action after bacterial expression

3. **Responsibilities:**

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4. **Required materials:**

   BugBuster® Protein Extraction Reagent (Millipore Sigma, USA)

   Expression plasmid (Gene of interest in pET-30a+) (GenScript, China)

   One Shot™ BL21(DE3) Chemically Competent E. coli (ThermoFisher, USA)

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

   Urea BioXtra, pH 7.5-9.5 (Sigma-Aldrich, USA)

   Imidazole, for molecular biology, ≥99% (titration) (Sigma-Aldrich, USA)

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

   Reduced glutathione (GSH) ≥98% (HPLC) (Sigma-Aldrich, USA)

   Oxidized glutathione (GSSG) ≥98% (HPLC) (Sigma-Aldrich, USA)

   Glucose ≥99.5% (GC) (Merck, USA)

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

   Glycerol, ReagentPlus®, ≥99.0% (GC) (Merck, USA)

   Prestained Protein Ladders (Sigma-Aldrich, USA)

   Plasmid Mini Kit (Promega, USA)

   HisTrap HP column (Millipore Sigma, USA)

   Amicon® Ultra Centrifugal Filters (Merck, USA)

5. Procedure:

Day 1-2

A. Protein expression and extraction

BL21 (DE3) *E. coli* cells were transformed with a pET30A+ expression vector encoding antigen or fragment of gene of interest with the codon-optimized sequence. Cells were grown to an OD_{600} of 0.5 and induced with 0.2 mM IPTG at 37°C.

B. Protein extraction by using a glass Dounce homogenizer

Step 1: Solubilization

After 2 h the cells from 1 L of culture were harvested at 10 000 rpm for 10 min and solubilized with 8 mL BugBuster reagent per liter of culture for 30 min at 30°C.

Step 2: Cell disruption by sonication

The volume of 15 mL of Base Buffer containing 20 mM Tris pH 8, 0.5 M NaCl, 10% glycerol and 5 mM β-mercaptoethanol, per liter of culture was added to the solubilized cells and sonicated on ice 15 × 20 s with a 50 % of duty cycle at 75 % of power. The resulting suspension was centrifuged for 10 min at 10 000 rpm at 4°C.

Step 3: Cell homogenization by glass Dounce homogenizator

The pellet was resuspended in 15 ml per liter of culture in Base Buffer supplemented with 2 M urea and homogenized with a glass dounce homogenizer. The sonication and centrifugation procedures were repeated, and the resulting pellet was resuspended by dounce in 15 mL per liter of culture of Base Buffer supplemented with 6 M urea. The pellet was again subjected to the same sonication and centrifugation procedures and the supernatant was collected.

C. On-column protein refolding

Day 3

The extracted supernatant was diluted 10-fold in Refolding Buffer containing 20 mM Tris pH 8, 20% glycerol, 55 mM glucose, 0.5 M NaCl, 2 mM reduced glutathione (GSH) and 0.2 mM oxidized glutathione (GSSG) supplemented with 2 M urea. The diluted extract was applied to a HisTrap HP column at 1 mL/min at room temperature. The column was washed with 2 column volumes (CV) of Refolding Buffer supplemented with 1.5 M urea at 0.2 mL/min for the first 0.5 CV and 0.6 mL/min for the remaining 1.5 CV. This procedure was repeated with Refolding Buffer supplemented with 1 M urea, 0.5 M urea and 0 M urea. Following the refolding procedure, the column was washed with Refolding Buffer supplemented with 30 mM imidazole and eluted with the same buffer supplemented with 0.5 M imidazole.

D. Gel filtration of refolded antigen

Day 4

In the final step of purification process, the protein fragment or antigen refolded on-column was loaded onto Superdex S200 column with length of 30 cm connected to AKTA Pure25 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 antigen obtained from the inclusion bodies was usually between 1.5 mg/L – 2.0 mg/L when the refolding procedure was performed on-column.

Note 1: Renaturation is a critical step in the recovery process of any biologically active protein from inclusion bodies. 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.

Note 2: *E. coli* expression system appears most suitable for the antigen screening purposes, providing an inexpensive and, most importantly, fast way to express, isolate, and analyze multiple protein fragments.

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