RUTGERS

Transcription Factor Antigen Production Protocol 8/1/2014

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Antigen Expression Protocol

1. Transform expression plasmid into *E. coli* BL21(DE3) or similar T7 expression cell line. Use of a rare tRNA helper plasmid (e.g. pMgK, pRARE2) may improve expression levels. We routinely use the lacY mutant Tuner(DE3)/pLysSRARE2 for expression.
2. Inoculate 1L expression culture with 1/100th volume overnight seed culture. Grow at 37°C with agitation (225 rpm) until OD_{600} reaches 0.6-0.8.
3. Lower growth temperature to 17°C and induce expression by the addition of IPTG to a final concentration of 0.1mM. Continue expression for 16h. (Note: Expression of some proteolytically sensitive constructs may be improved by continuing expression at 37°C after induction and harvesting after 3h.)
4. Harvest cells by centrifugation (20min @ 5000 x *g*, 4°C). Store cell pellet at -80°C.

Antigen Purification Protocol

Buffers

Solubilization Buffer: 50mM Tris-HCl, 6M urea, 10mM imidazole, 500mM NaCl, 1mM TCEP, 0.02% NaN_3, pH 7.5

Binding Buffer: 50mM Tris-HCl, 40mM imidazole, 100mM NaCl, 400mM L-arginine HCl, 20% glycerol, 1mM TCEP, (50µM $ZnSO_4$)*, 0.02% NaN_3, pH 7.5 * for zinc fingers

Elution Buffer: 50mM Tris-HCl, 500mM imidazole, 100mM NaCl, 400mM L-arginine HCl, 20% glycerol, 1mM TCEP, 0.02% NaN_3, pH 7.5

HEPES (GF) Buffer: 20mM HEPES, 100mM NaCl, 400mM L-arginine HCl, 20% glycerol, 1mM TCEP, 0.02% NaN_3, pH 7.5

Protocol

1. Thaw frozen cell pellets on ice and resuspend in 80ml solubilization buffer.
2. Lyse cells by sonication on wet ice (Sonicator 3000, sonication time: 10 minutes total, 5s on, 5s off, power level 7). Divide between two 50ml centrifuge tubes.
    a. Take aliquot of crude lysate as “total” protein fraction.
3. Clarify lysate by centrifugation (30min at 14,000 x *g*, 4°C) and transfer supernatant to clean centrifuge tubes.
    a. Take aliquot of clarified lysate as “soluble” protein fraction.
4. Overexpression of transcription factor domains in *E. coli*, especially as AviTag fusions, often results in inclusion body formation. The following protocol allows for recovery and purification of the antigen from inclusion bodies in high yield by solubilizing the entire cell pellet, binding to a HisTrap column in the presence of denaturant, and refolding the His-tagged antigens on the column prior to elution. Antigens can also be purified using a conventional HisTrap purification protocol if the soluble expression level is deemed sufficient.

a. Briefly, clarified lysate is loaded onto 5ml HisTrap HP columns pre-equilibrated in solubilization buffer. Columns are washed with solubilization buffer (5CV, 4ml/min), subjected to a slow refolding gradient from solubilization buffer to binding buffer (20CV gradient, 1ml/min flow rate), and eluted with a gradient from binding buffer to elution buffer (20CV gradient, 4ml/min). Target proteins typically elute at ≈150mM imidazole, and peaks are collected in 2ml fractions in a 96-well deep well plate.

5. HisTrap fractions and total/soluble aliquots checked for target protein by SDS-PAGE. Concentration and purity are estimated from the gel, and concentrations are confirmed by Nanodrop (A_{280}, using calculated extinction coefficients) and/or BCA assay.
6. Fractions of sufficient purity and concentration are pooled (and concentrated if necessary).
7. Samples requiring biotinylation are biotinylated *in vitro* according to the attached protocol. Extent of biotinylation is determined by MALDI-TOF MS of the TEV-cleaved tag, and biotinylation of target verified by pull-down assay using streptavidin magnetic beads.
8. Pooled and/or concentrated samples are injected into AKTAxpress for gel filtration (HiLoad 16/600 Superdex 75 pg column, equilibrated in HEPES buffer, flow rate 1ml/min, 1.2CV isocratic elution). Peaks are collected in 2ml fractions in a 96-well deep well plate.
9. Fractions are assayed by SDS-PAGE as described previously. The oligomeric state of the protein is estimated from the elution volume.
10. (Non-void volume) fractions containing target protein are pooled and concentrated, desalted, and/or dialyzed into appropriate buffer for shipment. Samples to be used for mouse immunization are supplied in a “ready to inject” format: dialyzed three times against PBS containing 20% glycerol to remove all arginine and sodium azide, and concentrated if necessary. QA/QC is performed on the dialyzed and concentrated samples to provide accurate dosing information.
11. Samples are stored frozen at -80°C prior to shipment on dry ice.

In-vitro Biotinylation Protocol

Biotinylation reactions are performed in pooled chromatography fractions (typical Avi-tagged antigen concentration: 2 - 120µM ≈ 0.04 – 1.5 mg/ml) in Elution Buffer (50mM Tris-HCl, 40-500mM imidazole, 100mM NaCl, 400mM L-arginine HCl, 20% glycerol, 1mM TCEP, 0.02% NaN_3, pH 7.5) or HEPES Buffer (20mM HEPES, 100mM NaCl, 400mM L-arginine HCl, 20% glycerol, 1mM TCEP, 0.02% NaN_3, pH 7.5).

Stock solutions:

BiomixA (10x stock): 0.5M bicine, pH 8.3

BiomixB (50x stock): 500mM MgOAc, 500mM ATP, 2.5mM d-biotin (prepare in 1M Tris base, adjust to pH 8.0 with concentrated NaOH)

1. Add BiomixA and BiomixB to Avi-tagged antigen (1x final concentration).
    a. The concentration of BirA (from a 1.2 mg/ml stock solution) used is dependent on sample concentration. In general, for samples <10µM, use 7.5µg BirA per 10nmole protein, 10µM to 25µM use 5µg BirA per 10nmole protein, and >25µM use 2.5µg BirA per 10nmole protein.
2. Incubate for 30 min at 30°C.
3. After biotinylation, samples are dialyzed or buffer exchanged by gel filtration to remove excess free biotin.
    a. Extent of biotinylation is determined by MALDI-TOF MS of the tag after TEV cleavage. Typically we see 100% biotinylation (only a peak corresponding to the mass of the biotinylated tag). Antigen biotinylation is also verified by a pull-down assay using streptavidin magnetic beads.