Induction and Purification of Biotinylated Fusion Protein

Reagents-Expression

Expression plasmid: pMal-T-Avi-His/BirA; 10 ng/ul in EB

 In the presence of IPTG and biotin, bacteria will generate MBP/biotinylated fusion protein.

Expression bacteria: NEB Shuffle T7 Express competent *E. coli* (#C3029)

Agar plates with ampicillin

Biotin (50x) solution: 2.5 mM Biotin (Sigma #B4639) in 10 mM Bicine, pH 8.3; store at -20d

 \*Most references suggest 5 mM stock solution; 2.5 mM is soluble at RT

IPTG (200x) solution: 100 mM IPTG in water, store at -20d

Ampicillin (1000x) solution: 100 mg/ml ampicillin in water; store at -20d

Reagents-Purification and Analysis

Amylose Resin: NEB (#E8021S); 50% bead slurry in 20% ethanol; store at 4d

TBS: Tris-buffered saline

Protease inhibitor (100x) stock solution: Pierce/Thermo HALT without EDTA; store at 4d

Maltose (5x) solution: 100 mM D-(+)-maltose (Sigma #M9171) in Factor Xa buffer; store at RT

Polyacrylamide gel filtration columns: Pierce/Thermo (#43426); 5 ml desalting columns; store at 4d

Factor Xa: NEB (#P8010S) 1 mg/ml; store at -20d

Factor Xa buffer: 20 mM Tris, pH 8.0 with 100 mM NaCl and 2 mM CaCl2

Factor Xa inhibitor EGR-CMK (1000x) solution: 10 mM in DMSO/DMF

Anti-MBP (maltose-binding protein) – Rabbit (EMD Millipore #AB3596) or Mouse (NEB # E8032S)

Streptavidin-Phosphatase – Jackson ImmunoReasearch (#016-050-084) diluted 1:5000

BCIP and NBT Alkaline phosphatase substrate and indicator: Pierce/Thermo diluted in DMF

Procedure – Induction/Biotinylation of MBP Fusion Protein

1) Thaw 50 ul aliquot of NEB Shuffle T7 bacteria on ice; add 20 ul to each of 2 pre-labeled tubes.

2) Add 1 ul of 10 ng/ul stock plasmid to bacteria, incubate on ice for 30 min.

3) Heat shock at 42d for 30 seconds, place tubes on ice for 1 min, then place in rack at RT.

4) Add 80 ul SOC media to each tube of bacteria.

5) Incubate bacteria at 32d in incubator for 1 h.

6a) Streak Amp plate with bacteria. Incubate plate at 32d overnight. The following afternoon, pick colonies and place in 3 ml LB + Amp. Grow overnight at 32d in shaking incubator.

6b) Alternatively, add entire 100 ul suspension to 3 ml LB + Amp solution and grow overnight.

7) The following morning, add 1 ml of bacteria to 2 ml pre-warmed (RT) LB + Amp. Return to 32d shaking incubator and grow for 2 h.

8) For each 3 ml culture, add 60 ul of 2.5 mM biotin stock solution and 15 ul of IPTG stock solution, return to shaking incubator for 3-5 h.

9) Add 1.5 ml bacteria suspension to 1.7 ml tube. Centrifuge at full speed for 1 min at RT. Discard supernatant. Add remaining 1.5 ml of bacteria to tube and repeat centrifugation.

10) Quick spin tubes and discard all supernatant. Add 250 ul TBS with HALT protease inhibitor.

11) Sonicate bacteria, place tube on ice.

12) Spin tubes 10 min at full speed at 4d.

13) Collect TBS-soluble solution; protein concentrations of the bacterial extracts should be around 10 mg/ml.

Blots: Dilute samples to 1.33 mg/ml, add 4x sample buffer and heat at 60d for 10 min. Electrophorese 10 ul samples in a 4-20% Bio-Rad acrylamide gel. A 10 ul aliquot represents approximately 10 ug of protein. Probe blots with anti-MBP (maltose binding protein) and streptavidin-phosphatase to confirm expression of the biotinylated fusion protein fusion protein.

Procedure – Batch Isolation of MBP/Biotinylated Fusion Protein

1) Gently shake bottle of amylose resin to make uniform suspension of beads.

2) Cut tip of P200 tip and place 200 ul suspension (100 ul beads) into 1.7 ml tube.

3) Add 1 ml TBS, mix, centrifuge, discard supernatant.

4) Repeat washes for a total of 4 times.

5) Add TBS-soluble bacterial extract to beads. Place on shaker at RT for 1-2 h.

6) Spin tubes and discard supernatant.

7) Wash 2x with 1 ml TBS, discard supernatant.

8) Wash 1x with 1ml Factor Xa buffer, discard supernatant.

9) Dilute 100 mM maltose solution 1:5 in factor Xa buffer. Add 200 ul of the 20 mM maltose solution to the beads. Periodically mix the beads with P200 tip. After 10-30 min, spin and collect supernatant. Note that trace amounts of beads will likely be present in the collected supernatant. Beads can be removed with a syringe filter or spin column. Protein concentrations should be about 500 ug/ml.

Blots: Dilute samples 1:5 (approximately 100 ng/ul), add 4x sample buffer and heat at 60d for 10 min. Electrophorese 10 ul samples in a 4-20% Bio-Rad acrylamide gel. A 10 ul aliquot represents approximately 0.5 ug of protein. Probe blots with anti-MBP (maltose binding protein) and streptavidin-phosphatase to confirm purification of biotinylated fusion protein.

Procedure – Cleavage of MBP/Fusion Protein with Factor Xa

1) Add 1.5 ul (1.5 ug) of Factor Xa to 200-250 ul of the MBP/biotinylated fusion protein solution, mix with pipette.

2) Incubate at RT for approximately 3 h. A time-course may have to be performed to determine the optimal incubation time. Depending on the downstream application, removing the maltose and calcium may not be necessary, especially if all of the fusion protein is cleaved. If the desalting step is necessary (e.g. remove calcium for binding to Ni or Co matrix), equilibrate polyacrylamide column with TBS (>6 ml) during this step.

3) Dilute EGR-CMK 1:10 in Xa buffer and add 2 ul to the digest solution (final conc. = 10 uM).

4) Add protein solution to 5 ml polyacrylamide column that has been previously equilibrated with TBS. Add 3-250 ul aliquots of TBS to the column (Vt = 1 ml). Continue to add 250 ul aliquots and collect ten fractions in 1.7 ml tubes. Protein will elute in the void volume which corresponds to approximately 2 ml (fractions 4 and 5). Protein concentrations of two or three fractions should be approximately 100 ug/ml.

5) Remove free MBP using amylose resin. Although this step will remove some MBP, it is not as efficient as the first pass. Batch equilibrate 100 ul (200 ul 1:2 slurry) of beads in 1.7 ml tube. Add “desalted” (maltose and calcium) protein, incubate for 1h at RT. Add bead solution to a spin column, centrifuge, and collect flow-through.

Blots: Utilize undiluted samples, add 4x sample buffer and heat at 60d for 10 min. Electrophorese 10 ul samples in a 4-20% Bio-Rad acrylamide gel. Probe blots with anti-MBP (maltose binding protein) and streptavidin-phosphatase to confirm cleavage of the MBP/biotinylated fusion protein fusion protein and estimate purity (relative to MBP).

(Optional) Procedure – Purification of the biotinylated fusion protein (His-tag)

At this point, the fusion protein solution contains biotinylated fusion protein, Factor Xa (inactive), and trace amounts of both MBP/biotinylated fusion protein and MBP. If the biotinylated fusion protein will be used in a streptavidin-based assay, and only trace amounts of uncleaved MBP/fusion protein are present, then this step is unnecessary.

Affinity purification of the biotinylated fusion protein with a cobalt metal matrix will remove the majority of contaminants. The pMal vector contains a His-tag after the AviTag, so only the biotinylated fusion protein will be immobilized (His-tag of BirA is not translated).

Most proteins can be eluted from the metal matrix with 250 mM imidazole. Some proteins can be eluted with pH 5.0 buffer. It may be necessary to compare elution conditions depending on downstream applications.