In vivo biotinylation protocol for AviTagged-proteins

Summary or abstract

Biotinylation of an antigen is often the method of choice for protein immobilization to select and evaluate affinity reagents. The tight and specific interaction of biotin with streptavidin or avidin is thereby used in many selection systems to specifically capture the antigen and/or affinity reagent for further analysis. In vitro biotinylation is frequently used, whereby lysine residues in the antigen are chemically modified. Biotinylation of a short acceptor peptide in vivo is an attractive method to achieve site-specific modification without the risk to interfere with protein folding or function of the antigen. In vivo biotinylation is achieved by co-expressing the protein of choice (fused to a biotin acceptor peptide) and the bacterial biotin-protein ligase, holocarboxylase synthetase (BirA) in the presence of biotin.

Overview of protein production method

The methods used for cloning, protein expression and purification are summarised briefly here; full details for expression of intracellular proteins have been published (1). Multiple constructs of target genes are cloned in parallel as PCR fragments, using ligation-independent cloning (LIC). The cloning vectors for E. coli include fusion tags for affinity purification, typically N-terminal His$_6$ tags that can be cleaved with Tobacco Etch Virus (TEV) protease. After clone verification, the plasmids are used to transform an expression strain, typically a derivative of Rosetta2 (a BL21 derivative harbouring the plasmid pRARE2 that provides 7 rare-codon tRNAs; Novagen). All clones are tested in small-scale cultures in rich medium (TB or LB), and protein expression is induced by IPTG at low temperatures (18°C). The recombinant proteins are then purified from clarified lysates by immobilised metal affinity chromatography (IMAC) in batch, and the eluted proteins are detected by SDS-PAGE. Selected clones are grown and induced to a larger scale (1–6 L) and the proteins are purified by protocols including IMAC, gel filtration and for some proteins tag cleavage and additional steps as indicated. Proteins are analyzed by SDS-PAGE and mass spectrometry. Variations of this basic procedure include the use of different fusion tags such as C-terminal His$_6$ tag, N-terminal His$_6$-thioredoxin tags (1), or biotin acceptor peptides (2).

As part of the preparation of antigens, we have designed and tested two expression vectors, pNIC-Bio3 and pNIC-Bio2 which are kanamycin-resistance vectors that express fusion proteins with N-terminal histidine tags (His$_6$ and His$_{10}$, respectively) followed by a TEV protease cleavage site, and a C-terminal biotin acceptor site. Both vectors are suitable for ligation-independent cloning as described (1). The Escherichia coli BirA gene, encoding biotin-protein ligase, was cloned into plasmid pCDF-DUET1 (Novagen; spectinomycin-resistance), creating the plasmid pCDF-BirA. The expression host strain BL21(DE3)-R3- Rosetta is a phage T1- resistant strain bearing a plasmid (pRARE2; chloramphenicol-resis-tance) that provides rare-codon tRNAs. This strain was transformed with pCDF-LIC and colonies were selected on media containing chloramphenicol (34 mg/ml) and spectinomycin (50 mg/ml) to create the strain Rosetta-R3-BirA, which was used as host in biotinylation experiments.

The plasmid sequences have been deposited with the following accession numbers:


Reagents

- Competent cells (E.Coli strains of BL21 (DE3) encoding the BirA gene for biotin-protein ligase)
- Luria-Bertani (LB) or Terrific broth (TB) Media
- Luria-Bertani (LB) Agar plates
Streptomycin (50mg/ml)
Chloramphenicol (34mg/ml)
Kanamycin (50mg/ml)
Isopropyl thio-β-D-galactoside (IPTG)
Bicine buffer pH 8.3
D-Biotin
Acrodisc 0.22μM & 0.45μM syringe filter (Minisart)
Hepes pH 7.5, 1M Stock solution
Sodium chloride (NaCl), 5M Stock solution
Imidazole, 3M Stock solution
Glycerol, 100% Stock solution
Tris(2-carboxyethyl)phosphine (TCEP), 1M Stock solution
Protease inhibitors cocktail set VII (Calbiochem, 1:1000 dilution)
Polyethylenimine (PEI), 5% stock at pH 7.4
Nickel-sepharose resin (GE Healthcare)
DL-Dithiothreitol (DTT), 1M Stock solution
Tobacco Etch Virus TEV protease
SnakeSkin dialysis tubing (Thermo Scientific)
Amicon-Ultra MWCO centrifugal concentrators (Millipore)

Equipment

Water Bath
Centrifuge 5810R (Eppendorf)
Centrifuge Avanti J-20XP (Beckman Coulter)
37°C Incubator
250ml / 2L Baffled flasks
Airporous Seals for Growing Cultures
Shaker-Incubators (Infor)
Visible light spectrometer for OD₆₀₀ measurement of bacterial cultures
High pressure cell homogeniser (the authors use either an Emulsiflex C5/C3 (Glen Creston, UK) or a Z model cell disrupter (Constant Systems, UK)
Sonicator (Sonics VibraCell)
Glass Econo-Column chromatography columns (BioRad)
Gel electrophoresis equipment for protein analysis
HPLC column, Electro spray Ionisation Mass Spectrometry – Time of flight (TOF) analyser (Agilent Technologies)
Size exclusion chromatography columns; HiLoad 16/60 Superdex S200 or Superdex S75 (GE Healthcare)
ÄKTA - Express or ÄKTA - Prime systems (GE Healthcare)
Nanodrop ND-1000 (NanoDrop Technologies) spectrophotometer or Bradford coomassie assay (BioRad)
10ml-Conical polypropylene columns (BioRad)
50ml-conical polypropylene tubes

Instructions

1. **A.** Bacterial transformation of target protein into a BL21 (DE3) / BirA co-expression strain: Steps 1 -3 Timing: 2 Days
2. **B.** Over-Expression and *in vivo* biotinylation of target protein: Steps 1 -9 Timing: 3 Days
3. **C.** Purification of biotinylated protein: Steps 1 – 7 Timing: 3 – 4 Days (dependent on chromatography columns and tag cleavage)

**A. Transformation of target gene into BL21 (DE3) / BirA co-expression strain**
1. Prepare fresh transformation of target plasmid DNA into *E. coli* BL21(DE3)- Rosetta-R3-BirA expression host strain. 2 - 6μl of miniprep plasmid DNA is used depending on concentration (100 – 400ng) and mixed with 40μl of Rosetta-R3-BirA competent cells.

2. Plate the transformation on LB agar plate containing the appropriate antibiotics, e.g. LB agar containing Chloramphenicol 34mg/ml (for BL21(DE3)-R3-pRARE2), Streptomycin 50mg/ml (for pCDF-BirA) and kanamycin 50 mg/ml (for plasmid expression pNIC-Bio3 or Bio2).

3. Incubate LB agar plate(s) overnight at 37°C.

### B. Over-Expression and in vivo biotinylation of target protein

1. **Starter cultures.** Pick several colonies from the overnight transformation and use to inoculate 10-30ml LB medium in a 250ml baffled flask, supplemented with Streptomycin (50mg/ml), Chloramphenicol (34mg/ml), and Kanamycin (50mg/ml).

2. Grow overnight in a 37°C incubator with shaking at 200 – 250 rpm.

3. **Protein expression.** The next day. Prepare 1L of LB or TB media in a 2L baffled flask supplemented with Streptomycin (50mg/ml) and Kanamycin (50mg/ml), inoculate the medium with 10ml from the overnight starter culture. Seal flask with an adhesive airporous film during cell growth. Note: the medium need not contain chloramphenicol at this step; the pRARE2 plasmid is not lost during the growth process and also ensures a more predictable growth.

4. Grow culture in a 37°C incubator with shaking at 190 – 220 rpm. Monitor cell growth until Mid-log phase is reached and cell density OD600nm is approximately 0.5 - 0.7 in LB or 2.00 – 3.00 in TB. Shift the temperature of the shaker to 18ºC and allow culture flask to cool.

5. After 30 – 60 minutes, induce expression with the addition of IPTG. During the induction process Biotin solution is added in appropriate concentrations. We have observed that biotinylation efficiency can vary from protein to protein, therefore induction of IPTG and Biotin concentrations may need to be optimised for specific proteins. Highly expressing proteins generally require less IPTG concentration than lower expressing proteins. Cultures are induced with 0.1mM – 0.5mM IPTG and 0.1mM – 0.3mM Biotin solution. These concentrations have been tested on kinases and phosphatases and have shown to produce 80 – 100% biotinylation of the protein. Example induction: 0.1mM (100uM) IPTG ; 0.1mM (100uM) Biotin. Add 10 ml of 10mM biotin solution (100 μm final). The biotin solution is made by adding 24mg of d-biotin to 10 ml of warm (microwaved) 10 mM bicine buffer (pH 8.3) and filter sterilisation of the solution with a syringe and a 0.2 micron filter. 10ml of biotin solution is added to each 1L culture on induction with IPTG.

6. Continue incubation of culture for ~6 hours or incubate overnight at 18°C.

7. **Cell collection.** Cultured cells are collected by centrifugation using a JLA 8.1 fixed-angle rotor, centrifuged for 20 minutes at 6000rpm and 4°C.

8. Discard supernatant; using a rubber spatula transfer cell pellet into a 50ml conical polypropylene tube. Cell pellets may be stored at -20°C or processed immediately.

9. **Cell Lysis.** For frozen cells, remove from -20°C and allow cell pellet to thaw at room temperature or by intermittently placing in a 37°C water bath. Transfer thawed pellet directly to ice. Re-suspend the pellet in pre-chilled lysis buffer by vortex or pipetting gentle until cells are completely in suspension. Lysis buffer; 50mM Hepes pH 7.5, 500mM NaCl, 5mM Imidazole, 5% Glycerol, 1mM TCEP, 1x protease inhibitors (1/1000 dilution). Cell suspensions are disrupted by sonication on ice or by high pressure homogenisation (25 kpsi).

  - Homogenisation: cell samples are passed through a homogeniser 3-4 times or according to the equipment instructions and the lysate then transferred to a 50ml Beckman centrifuge tube. Sonication: transfer the cell suspension to a suitable tube or beaker on ice, the author’s use a Sonics VibraCell set at 35% amplitude with pulses of 5 seconds on and 10 seconds off for a total time of 5 – 10 minutes. The lysate is then transferred to a 50ml Beckman centrifuge tube. Cell debris and DNA in the lysate is precipitated by adding PEI to the tube from a 5% stock solution to a final concentration of 0.15% (30μl/ml of lysate). Cell lysates are clarified by centrifugation using a JA25.50 rotor at 21,500rpm for 60 min at 4°C or a JA.17 rotor at 17,000rpm. Collect the cleared supernatant for purification and filter using a 0.45um acrodisc filter using a 30ml syringe.
C. Purification of biotinylated protein

1. Ni²⁺-NTA Agarose gravity flow chromatography (column 1). Column preparation: Wash a 25ml glass econo-column with water. To the column add 1-2ml of packed nickel resin per 1L of culture (or adjust volume dependent on the estimated level of protein expression), wash resin 2-3 times in water, equilibrate the resin in 10 column volumes of binding buffer; 50mM Hepes pH 7.5, 5% glycerol, 500mM NaCl, 5mM imidazole, 0.5mM TCEP. **The remainder of this step should be carried out at 4°C.** Load the cell lysate onto the column and pass through resin at gravity flow, collect the unbound flow-through, wash column with 20 column volumes of wash buffer; 50mM Hepes pH 7.5, 5% glycerol, 500mM NaCl, 30mM imidazole, 0.5mM TCEP, collect the wash fraction, elute the protein in 5 intermediate fractions with a gradient of 5ml imidazole elution buffers; 50mM Hepes pH 7.5, 5% glycerol, 500mM NaCl, 0.5mM TCEP, 5mM, 100mM, 150mM, 250mM (x2) imidazole, respectively, collect each gradient eluted fraction. Analyse eluted Ni²⁺-NTA fractions by SDS-PAGE for identification of recombinant antigen

2. Load 10µl of unbound flow-through, wash fraction, and eluted fractions onto SDS-PAGE gel; analyse fractions for expected mass and purity. Pool together selected eluted protein fractions.

3. Identity and biotinylation efficiency is verified by mass spectrometry, the authors use Electrospray Ionisation Mass Spectrometry – Time of flight (TOF) analyser, Biotin quantitation is measured by: MW of biotinylated protein = MW of sequenced Protein + 226 Da (biotinylation modification).

4. Size Exclusion Chromatography (SEC) (Column 2). Concentrate pooled Ni²⁺-NTA protein fractions to required concentration and volume for gel filtration, using an Amicon-Ultra MWCO centrifugal concentrator. Equilibrate the column in gel filtration buffer. The authors use a Superdex S200 16/60 HiLoad, 120ml on an ÄKTA Prime chromatography system equilibrated in 50mM Hepes pH 7.5, 300mM NaCl, 0.5mM TCEP. Load protein onto the GF column for further purification; protein is fractionated on the column in GF buffer at 1ml/min and 1.75ml fractions are collected at the A280 peaks. Analyse the peak fractions by loading 10µl of each collected fraction onto SDS-PAGE gel. Collect and pool relevant column fractions, selecting fractions containing high protein concentration and purity, eliminating any bound contaminating non-specific proteins. Further purification may be necessary dependent on required purity of the protein, otherwise protein concentration is assessed by measuring the absorbance at 280nm or by Bradford assay (Step 7). Preferable the protein should be as pure and homogeneous as possible for subsequent experiments. Further purification steps using chromatographic methods, e.g. Ion exchange, may be applied or overnight digestion with Tev protease to remove the terminal histidine tag may be an effective method for removing contaminating proteins when passed over a Ni²⁺-NTA re-binding column (Step 6).

5. Enzymatic treatment of tag (Optional). To perform cleavage of the histidine tag, add Tev protease to a 1:20mg/ml ratio, store protein sample containing Tev at 4°C overnight for digestion. The next day apply protein to a Ni-NTA re-binding column. Note: Enzymatic treatment with Tev protease should be carried out on proteins containing no imidazole in the storage buffer, i.e. after gel filtration. High Imidazole concentrations as in elution buffers inhibit the cleavage process.

6. Ni²⁺-NTA Rebinding column (Column 3). Separate cleaved protein from any un-cleaved protein by passing over a Ni²⁺-NTA column. Cleaved protein should not bind the nickel and may be recovered in the flow-through fractions. Non-specific contaminating proteins with any un-cleaved protein will bind to the column and can be eluted with imidazole. To a 10ml-polypropylene column add ~0.5 - 1ml packed nickel resin, wash resin in 10 column volumes of water then equilibrate in 10 column volumes of binding buffer; 50mM Hepes pH 7.5, 5% glycerol, 500mM NaCl, 5mM imidazole, 0.5mM TCEP. Load the protein plus Tev protein sample and allow to pass through column by gravity flow. Collect the flow-through, to wash any remaining protein from the column include a 2X wash step column volume of gel filtration buffer; 50mM Hepes pH 7.5, 300mM NaCl, 0.5mM TCEP, to elute any bound protein apply a column volume of imidazole elution buffers, e.g. containing gradients of 30mM, 100mM, 200mM and 300mM imidazole. Load a 10µl sample from each collected fraction onto
SDS-PAGE gel and analyse for verification of cleaved protein and purity.

7. **Protein concentration mg/ml and storage.** Concentrate protein to the required concentration/volume using an Amicon-Ultra MWCO centrifugal concentrator. Final protein concentration is determined either from the absorbance at 280nm, measured using a nanodrop spectrometer or by performing a Bradford reaction assay. Storage; Flash-freeze the protein in small aliquots in liquid nitrogen, adding 5% glycerol to the final protein concentration prior to freezing may improve the stability of the protein during the later thawing process. Store protein at -80°C.

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**Citation**


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