Site-specific biotinylation of purified proteins using BirA

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Summary

The binding between biotin and streptavidin or avidin is one of the strongest known non-covalent biological interactions. The (strept)avidin-biotin interaction has been widely used for decades in biological research and biotechnology. Therefore labeling of purified proteins by biotin is a powerful way to achieve protein capture, immobilization, and functionalization, as well as multimerizing or bridging molecules. Chemical biotinylation often generates heterogeneous products, which may have impaired function. Enzymatic biotinylation with *E. coli* biotin ligase (BirA) is highly specific in covalently attaching biotin to the 15 amino acid AviTag peptide, giving a homogeneous product with high yield. AviTag can conveniently be added genetically at the N-terminus, C-terminus or in exposed loops of a target protein. We describe here procedures for AviTag insertion by inverse PCR, purification of BirA fused to glutathione-S-transferase (GST-BirA) from *E. coli*, BirA biotinylation of purified protein, and gel-shift analysis by SDS-PAGE to quantify the extent of biotinylation.

Key words: Neutravidin, Streptavidin-biotin, Femtomolar, Nanotechnology, Bionanotechnology

Running Head: BirA biotinylation

1. Introduction

Biotin is a cofactor for carboxylase enzymes, present in all living organisms (1). Streptavidin binds to biotin with a K_d of 4×10^{-14} M (2). Streptavidin-biotin binding is rapid, specific and can still occur under conditions where most other proteins have denatured, such as high temperatures or 6 M guanidinium hydrochloride or 1% sodium dodecyl sulfate (SDS) (3). A breakthrough for the use of biotin for protein modification was harnessing the cell's natural machinery for biotin conjugation, using the *E. coli* enzyme BirA to achieve precise biotin modification (4). The natural substrate of BirA is the Biotin Carboxyl Carrier Protein (BCCP), requiring fusion of at least 75 residues to the target protein (4). However, phage display selection enabled the development of the AviTag (also known as the Acceptor Peptide, AP), which is superior to BCCP as a BirA substrate but only 15 amino acids in length (5) (*see* **Fig. 1**), so extending the range of protein sites amenable to site-specific enzymatic biotinylation.

Fig.1. Principle of BirA use. (**A**) Biotin ligase (BirA) reaction, covalently linking free biotin to the lysine of AviTag. (**B**) Advantage of labeling with BirA compared to labeling with amine-reactive biotin N-hydroxysuccinimide (NHS) esters, illustrated with regard to a Fab antibody fragment.

More recent work has established that BirA can biotinylate such substrate peptides specifically in the cytosol (6), secretory pathway, and at the cell surface in mammalian and invertebrate systems (7,8,9,10). A detailed protocol for labeling with BirA at the mammalian cell surface for fluorescent imaging has recently been published (11).

Biotinylation of purified proteins has been applied in a wide range of areas of biochemistry and cell biology (*see* **Fig. 2**):

• Tetramerization - enhancing the avidity of ligand binding. For example, MHC class I tetramerized by streptavidin enabled stable binding to the T cell receptor and so allowed monitoring of the immune response and isolation of anti-pathogen or anti-cancer T cells (12).

• Bridging - for nanoassembly, streptavidin is often used as a bridge between one biotinylated protein and another biotinylated molecule, such as DNA, sugars, lipids or small-molecule drugs (13).

• Immobilization - giving precise attachment that is stable over time, to a wide range of pH values, and to force. BirA-biotinylated proteins are commonly used for capture on chromatography columns, chips (e.g. for surface plasmon resonance or next generation sequencing) (14), atomic force microscope tips (15), or nanoparticles (e.g. quantum dots or magnetic particles) (16).

• Sensitive detection - an *in vitro* biotinylated protein can be added to cells and subsequently recognized with high affinity by streptavidin conjugates (17). Use of monovalent streptavidin facilitates efficient measurement of the absolute number of biotin binding sites on cells (16).

Fig. 2. Common applications of BirA biotinylation of purified proteins.

Further developments

An important advance in BirA labeling is its use for electron microscopy (18). Biotin ligase from *E. coli* or other species can also ligate to a peptide tag biotin analogs,

including desthiobiotin for reversible streptavidin binding (19), or analogs containing functional groups for bio-orthogonal reaction: keto (20), azido and alkyne groups (21). However, only small changes to the structure of biotin could be tolerated by biotin ligase and so the related ligase LplA has proved more amenable to direct incorporation of fluorophores (22).

Engineering of streptavidin is important in extending the usefulness of BirA-labeling; in particular variants with controlled valency (e.g. monovalent streptavidin, mSA), enabling precise control over assembly of biotin conjugates (11,23). In addition, we generated a streptavidin variant with 10-fold lower off-rate for biotin and enhanced thermal stability (traptavidin) (24,25).

New applications of BirA have been for labeling specific protein populations – by targeting BirA to a specific chromatin-associated protein, particular AviTag-linked nucleosome populations were biotinylated (26). By targeting BirA to one synaptic membrane, AviTag-proteins on the opposite synaptic membrane were biotinylated, allowing imaging of specific protein-protein interactions at synapses (27). Through expressing a BirA-substrate peptide on a nuclear envelope protein and BirA in specific tissues of *Arabidopsis thaliana*, *Caenorhabditis elegans* or *Drosophila melanogaster*, nuclei from specific cell-types could be isolated by streptavidin pull-down (28,29). Also the use of enzymes to achieve *promiscuous* biotinylation (a BirA mutant or a peroxidase) has enabled labeling of untagged proteins in particular cellular regions or compartments (30,31,32).

Limits of BirA protein labeling

The convenience and high yield of BirA labeling must be considered against certain limitations:

• a peptide tag must be introduced into the target protein.

For site-specific biotinylation while only changing a single residue, suppressor tRNA bearing a biotinylated amino acid can be used (although some protein locations were not well tolerated) (33). However, biotinylation via artificial amino acid incorporation brings disadvantages of more complex expression and of uncertainty in percentage incorporation- the initial assessment of biotinylation yield in *Xenopus* oocytes was done indirectly via electrophysiology and radioactive streptavidin binding (33). p-Aminophenylalanine-linked biotin conjugates on tRNA showed improved protein incorporation in cell-free translation (34) (reagents are available from RiNA GmbH or Cosmo Bio Co. Ltd.). Biotinylation can also be achieved directly at the N-terminus, such as with subtiligase (35), or at the C-terminus using inteins (36).

• the binding partner of biotin, streptavidin or avidin, does not interact covalently and is not a good fusion partner.

Covalent linkage to peptide tags can now be achieved using split inteins (37,38), sortase (39) and SpyCatcher (40), although they have not yet demonstrated the high sensitivity of detection shown by streptavidin or avidin. A monomeric streptavidin has been developed that is suitable as a fusion tag (41). A key future development will be to improve monomeric streptavidin's binding affinity to that of the original tetrameric streptavidin.

2. Materials

2.1 Equipment

1. Incubators and shakers appropriate for growing bacterial cultures.

2. Centrifuges: floor-standing centrifuge capable of spinning at 5,000 g on 1 L bacterial culture and bench-top centrifuge capable of spinning 1.5 mL tubes at 20,000 g.
3. Sonicator or other cell-disrupting apparatus (e.g. French press).
4. UV-Vis spectrophotometer or Nanodrop for protein quantification.
5. Electrophoresis apparatus for running SDS-PAGE.
6. PCR machine.

2.2 Proteins, DNA and other Reagents

1. Streptavidin (commercially available from several sources including Thermo Scientific, Sigma and Roche) (*see* **Note 1**).
2. pGEX-GST-BirA plasmid (42) (a kind gift from Chris O'Callaghan, University of Oxford); alternative expression vectors containing Maltose Binding Protein-BirA or His_6-BirA are available through Addgene (www.addgene.org).
3. Complete Protease Inhibitor Cocktail tablets (Roche) for inhibiting *E. coli* proteases during purification of GST-BirA.
4. 100 mM PMSF solution: 17.4 mg of phenylmethylsulfonyl fluoride (PMSF) in 1 mL of isopropanol. Store at -20 °C. (CAUTION: PMSF is toxic. PMSF should be added to the aqueous buffer just prior to use, as it has a short half-life in aqueous solutions.)
5. Glutathione-HiCap resin for purification of GST-tagged proteins (Qiagen).
6. Target protein with AviTag peptide sequence (*see* **Note 2**).
7. KOD hot start polymerase (Merck Millipore)
8. T4 DNA ligase (NEB)
9. T4 polynucleotide kinase (NEB)

10. DpnI (NEB).
11. 50 mM D-Biotin solution: 12.2 mg of D-biotin in 1 mL of anhydrous DMSO. Store at -20 °C.
12. 100 mM ATP solution: 55.1 mg of adenosine 5'-triphosphate disodium salt hydrate in 1 mL MilliQ water. Store in aliquots at -80 °C (*see* **Note 3**).
13. 1 M magnesium chloride solution: 203 mg of magnesium chloride hexahydrate in 1 mL MilliQ water. Store at room temperature.
14. 100 mM DTT solution: 15.4 mg of dithiothreitol in 1 mL MilliQ water. Aliquot and store at -20 °C. Make freshly each week.
15. 420 mM IPTG solution: 1 g isopropyl-β-D-thiogalactopyranoside in 10 mL MilliQ water. Syringe-filter and store at -20 °C.
16. 100 mg/mL Ampicillin solution: 1 g of ampicillin sodium salt in 10 mL MilliQ water. Syringe-filter and store at -20 °C.
17. 20% glucose solution: 200 g/L of D-glucose in MilliQ water. Autoclave and store at room temperature.

2.3 Buffers, Media and Cells

1. Phosphate buffered saline (PBS): 1.44 g/L di-sodium hydrogen phosphate, 0.24 g/L potassium di-hydrogen phosphate, 0.2 g/L KCl, 8 g/L NaCl, pH 7.4.
2. PBS-L buffer: PBS, 1 mM EDTA, 1 mM DTT, 0.1 mg/mL lysozyme, 1% Triton X-100 (make fresh each day).
3. PBS-EW buffer: PBS, 1 mM DTT and 1 mM EDTA.
4. Elution buffer: 50 mM Tris.HCl pH 8.0, 0.4 M NaCl, 50 mM reduced glutathione, 1 mM DTT. Make this buffer fresh on each occasion.

5. Luria Bertani broth (LB): 10 g/L bacto-tryptone, 5 g/L yeast extract, 5 g/L NaCl. Autoclave and store at room temperature.
6. *E. coli* strain suitable for protein expression, e.g. BL21 [DE3] RIPL (Agilent).
7. 2× SDS-PAGE buffer (non-reducing): 4% SDS, 20% glycerol, 0.12 M Tris.HCl pH 6.8. Store aliquots at -20 °C.

3. Methods

The methods described below utilize a glutathione-S-transferase-BirA fusion protein, but are adaptable to His_6-tagged or Maltose Binding Protein (MBP) fusion constructs. All three constructs express well but GST-BirA can be efficiently removed from the biotinylated substrate after reaction, by passing through glutathione-agarose.

3.1 GST-BirA production

1. Transform an appropriate *E.coli* expression strain (e.g. BL21) with the pGEX-GST-BirA plasmid.
2. Grow a 10 mL overnight culture from a single colony in LB plus 10 μL of 100 mg/mL ampicillin and 200 μL of 20% glucose.
3. Use 8 mL of the overnight culture to inoculate 800 mL LB plus 0.8 mL 100 mg/mL ampicillin and 30 mL 20% glucose in a 2 L baffled flask.
4. Grow at 37 °C with 200 rpm shaking to an OD_{600} of 0.5.
5. Induce protein expression by addition of 0.8 mL of 420 mM IPTG solution.
6. Continue growth at 25 °C with 200 rpm shaking overnight.
7. Harvest cells by centrifugation for 10 minutes at 5,000 g at 4 °C.
8. Resuspend cells in 15 mL of PBS and freeze at -80 °C.

9. Thaw cells on ice and add 0.17 mL of 10 mg/mL lysozyme, one Complete Protease Inhibitor Cocktail tablet, 0.17 mL of 100 mM PMSF, 1.7 mL of 10% Triton X-100, 0.17 mL of 100 mM EDTA and 0.17 mL of 100 mM DTT.
10. Incubate 30 minutes on ice and freeze again at -80 °C to help cell lysis.
11. Thaw cells and add 15 mL cold PBS-L buffer. Hereafter, keep the sample at 4 ºC at all stages.
12. Sonicate to reduce viscosity (e.g. 3-5× 30 second bursts on ice). (CAUTION: wear appropriate ear protection.)
13. Centrifuge lysed cells at 20,000 g for 30 minutes.
14. Collect the supernatant and add 1 mL of glutathione-HiCap resin to the supernatant, mixing end-over-end for 30 minutes at 4 °C.
15. Centrifuge resin for 2 minutes at 1,000 g and discard supernatant.
16. Wash resin with 30 mL PBS-EW. Centrifuge resin for 2 minutes at 1,000 g and repeat the wash.
17. Elute GST-BirA with 2 mL Elution buffer and incubate for 30 minutes at 4 °C.
18. Centrifuge resin for 2 minutes at 1,000 g and collect supernatant.
19. Check purity by SDS-PAGE (14% polyacrylamide) (*see* **Fig. 3**) and concentration via OD_{280} (GST-BirA has an ε_{280} of 90,550 $M^{-1}cm^{-1}$).
20. Concentrate by ultrafiltration to ~50 µM and store in single-use aliquots at -80 °C. Concentrations of GST-BirA much greater than 50 µM may crash out. Final yield should be 10-20 mg/L of expression culture. After thawing, aliquots stored at 4 °C should be used within 1 week.

Fig. 3. Expression and purification of GST-BirA. 14% SDS-PAGE with Coomassie staining of samples of the lysate (Lys) and soluble fraction (Sol) of *E. coli* expressing

GST-BirA and varying amounts of the protein preparation purified with glutathione-resin.

3.2 Generation of AviTag protein constructs

A variety of standard molecular biology methods can be used to add the AviTag (*see* **Note 2**) to an appropriate site in a target protein (*see* **Note 4**). For certain experiments it may also be valuable to clone a negative control peptide that is not biotinylated by BirA (*see* **Note 5**). We suggest using a modified inverse PCR mutagenesis (43) (*see* **Fig. 4**) or Site-directed Ligase-Independent Mutagenesis (SLIM) reaction (44), which enables the insertion of the substrate peptide without requiring any restriction sites nearby. Below is an example inverse PCR mutagenesis protocol.

1. Forward and reverse primers for peptide insertion should be designed to each have 18-25 bp matching the parental sequence and have a calculated annealing temperature (to the parent sequence) of at least 55 °C (*see* **Fig. 4**).
2. Assemble the following reaction mixture in a PCR tube: 29.5 µL MilliQ water, 1.5 µL DMSO, 5 µL KOD polymerase buffer, 5 µL 25 mM $MgSO_4$, 1 µL 15 µM forward primer, 1 µL 15 µM reverse primer, 1 µL 100 ng/µL template plasmid DNA, 5 µL 2 mM dNTP mix and finally 1 µL KOD hot start polymerase.
3. After transferring the tube to a PCR machine, perform an initial denaturing step of 3 minutes at 95 °C, followed by 12 cycles of:

   95 °C for 30 seconds, 55 °C for 30 seconds and 68 °C for 30 seconds/kb of target plasmid DNA.
4. Add 1 µL of 20 U/µL DpnI enzyme to the PCR mix and incubate at 37 °C for 1 hour.

5. Run an aliquot of the reaction on a 0.7% agarose gel to confirm the success and fidelity of the PCR (a clean band should be observed corresponding to the size of the linearized target plasmid DNA).
6. To 2 μL of the PCR product, add 14 μL MilliQ water, followed by 2 μL of 10× T4 DNA ligase buffer, 1 μL T4 polynucleotide kinase and 1 μL of T4 DNA ligase.
7. Incubate the sample for 1 hour at room temperature and transform an appropriate strain of competent *E. coli* (e.g. DH5α, XL1-Blue, JM109) with 5 μL of the ligation reaction. Cells with competency of at least 10^7 cfu/μg should be sufficient.
8. After validating the construct by sequencing, the AviTag-fused protein can be overexpressed in the appropriate cell system (commonly *E. coli*, baculovirus or HEK 293T cells).

Fig. 4. Design of primers for AviTag insertion using the inverse PCR mutagenesis method.

3.3 Biotinylation of AviTag-fused proteins using BirA

1. To 100 μM AviTag-fused protein in 952 μL of PBS, add 5 μL 1 M magnesium chloride, 20 μL 100 mM ATP, 20 μL 50 μM GST-BirA and 3 μL 50 mM D-Biotin (*see* **Note 6**).
2. Incubate sample for 1 hour at 30 °C with gentle mixing on a rocking platform.
3. Add the same amount of fresh biotin and GST-BirA and incubate for a further hour.

4. GST-BirA may be removed by incubation of the sample with 0.1 mL of a 50% slurry of glutathione-HiCap resin in PBS for 30 minutes at room temperature, followed by centrifugation and collection of the supernatant (45).
5. Dialyze the sample into PBS or other suitable buffer, for storage and to remove the excess biotin.
6. The biotinylation of the target protein is generally irreversible *in vitro*; apparent loss of biotinylation is most likely to reflect proteolysis separating the biotinylation site from the rest of the target protein.

3.4 Testing the extent of protein biotinylation by a streptavidin gel-shift

The efficiency of the biotinylation reaction has been examined by Western blotting (6) or other enzymatic or ligand-displacement assays (46), but these approaches are time-consuming and only indirectly allow quantitation. A rapid and easily quantified alternative is to saturate the target protein with streptavidin and study the gel-shift in SDS-PAGE (*see* **Fig. 5**). Provided the gel does not get excessively warm during the run, streptavidin will retain its native tetramer structure and remain bound to biotin conjugates under normal SDS-PAGE conditions (16). A streptavidin monomer (i.e. one biotin binding site) has a calculated ε_{280} of 41,940 $M^{-1}cm^{-1}$.

1. Prepare a PCR tube containing 5 μL of 10 μM biotinylated target protein and add 10 μL of 2× SDS-PAGE buffer.
2. Heat samples at 95 °C for 5 minutes in a PCR block with a heated lid.
3. Allow the sample to cool to room temperature and briefly centrifuge.
4. **After** this boiling and cooling, add 5 μL of PBS containing a small molar excess (2- to 5-fold) of streptavidin to the samples and incubate at room

temperature for 5 minutes (it is advisable to run a control lane of streptavidin without the target protein).

5. Run samples on an appropriate SDS-PAGE gel (the streptavidin tetramer, running at 50-60 kDa, is clearly visible on 10, 12, 14, 16 % gels) (*see* **Note 7**).
6. Stain the gel with InstantBlue or Coomassie blue and visualize. If desired, quantify the degree of biotinylation by densitometry, measuring the change in intensity of the relevant protein band with and without addition of streptavidin (*see* **Note 8**). In the lane containing biotinylated protein and streptavidin, the presence of a band corresponding to free streptavidin verifies that streptavidin was indeed provided in excess and so all biotinylated protein will have been bound. Streptavidin may sometimes increase in mobility upon binding to biotin conjugates, according to the size and charge of the biotin conjugate (*see* **Fig. 5**).

Fig. 5. Testing the extent of biotinylation by SDS-PAGE gel-shift. Coomassie-stained SDS-PAGE of an antibody fragment (Fab0.35) with an AviTag on the C-terminus of both the heavy and light chains. The lanes represent non-biotinylated Fab (nb), biotinylated Fab, biotinylated Fab with streptavidin (SA), and streptavidin alone. Streptavidin has 4 binding sites and so may associate with 1 or 2 chains of the biotinylated target, but this does not affect the calculation of the depletion of the original target protein band.

4. Notes

1. Instead of streptavidin, other high affinity biotin-binding proteins may be used to bind to enzymatically biotinylated proteins. Avidin is not recommended because its positive charge promotes non-specific binding to cells and DNA, but neutravidin should be satisfactory for many applications (47).
2. Several peptide sequences have been described for BirA-mediated biotinylation. These are based on those first described by Schatz and

coworkers (48,5), who found a 13 amino acid peptide to be the minimal substrate peptide for BirA (LX§IFEAQ**K**IEWR, where X = any and § = any but not L, V, I, W, F or Y). This sequence was further optimized to improve the rate of biotinylation, resulting in AviTag (GLNDIFEAQ**K**IEWHE). AviTag works at either the N or C terminus of the target protein (46). A close 15 residue relative, termed BioTag (ALNDIFEAQ**K**IEWHA), is also used in some papers (48,10). BLRP (Biotin ligase recognition peptide) contains a core of AviTag and is 23 residues: (MAGGLNDIFEAQ**K**IEWHEDTGGS) (5,49). Another popular target is the 15 residue "BirA Substrate Peptide" (BSP), LHHILDAQ**K**MVWNHR (48,42). A further consideration is whether some flexibility should be added between the AviTag and the target protein. We would suggest including a flexible two residue GS linker between the AviTag and the target protein or any other surrounding peptide tag or domain. In the unlikely event that constructs with N-terminal or C-terminal AviTag do not enable biotinylation or yield low amounts of protein, first try increasing the spacer to 6 residues and then it may be worth trying BSP (42). Vectors are also available containing N- or C-terminal AviTag sequences from Avidity or from Genecopoeia (for bacterial, mammalian or cell-free expression; some plasmids have BirA downstream for coexpression).

3. Prepare single-use aliquots: freeze–thawing damages ATP stocks. Also, ATP will be hydrolyzed at pH greater than 8.5.
4. Selected examples of successful biotinylation following BirA-substrate peptide insertion in protein loops: the *E. coli* flagellar hook (50), Cystic Fibrosis Transmembrane Regulator (CFTR) (51), and Dicer (52). For Dicer, Lau et al. use streptavidin to highlight features for Cryoelectron Microscopy

and describe several functional and some non-functional peptide insertion sites, advising insertion in short loops disordered in the crystal structure or less highly conserved (52).

5. The Lys to Ala mutant of AviTag (GLNDIFEAQ**A**IEWHE) serves as an effective negative control sequence that will not be biotinylated (20,8). Note that AviTag-fusions expressed in *E. coli* may have some biotinylation from the cell's own BirA, but this reaction may often not reach completion, even in strains with BirA overexpressed (AVB101, Avidity) (53). Also, adding BirA to an AviTag-fusion in the absence of ATP or biotin may still allow some biotinylation to take place, because of biotin-AMP pre-bound to the purified protein (8,20).
6. Other buffers may be used for biotinylation. Schatz et al. prefer 50 mM bicine pH 8.3, maintaining low [NaCl] (46), but in our hands biotinylation in PBS is still quantitative. It is preferable to have the AviTag-fusion at concentration > 40 µM when incubating with BirA; otherwise biotinylation is less efficient (46). The biotinylation reaction may be run on a smaller scale; the only issue is that losses from dialysis become more significant when working with a low total amount of protein.
7. If your target protein happens to have exactly the same mobility as streptavidin, use a different percentage gel. The target protein is unfolded and will run according to its molecular weight, but streptavidin remains folded and runs at a different height on different percentage gels.
8. With incomplete biotinylation, it is possible to purify the biotinylated fraction using monomeric avidin (a chemically modified version of avidin with

reversible biotin binding) (45), but we would suggest that it is preferable to modify the biotinylation reaction until the reaction does go to completion.

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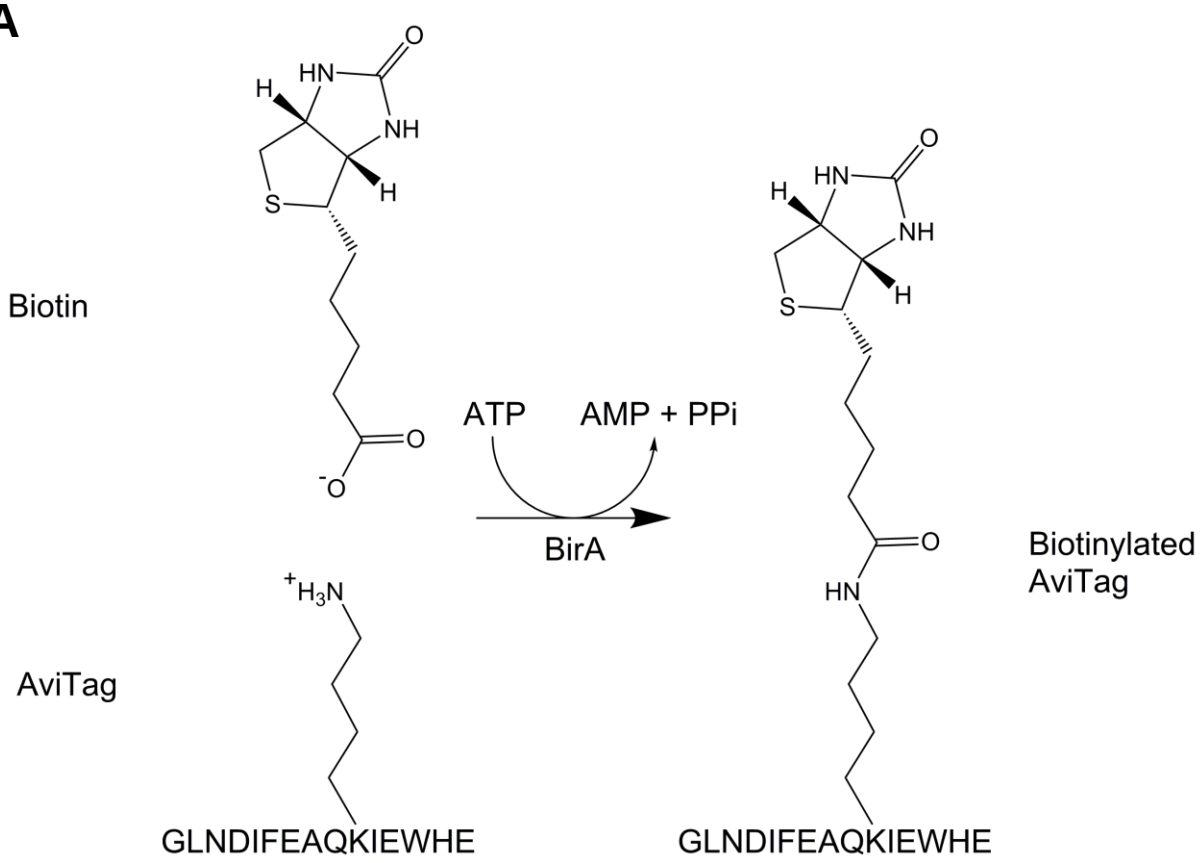
Figure 1

A



B

Fab: ~24 Lysines + 2 N-termini

↓ *Biotin-NHS*

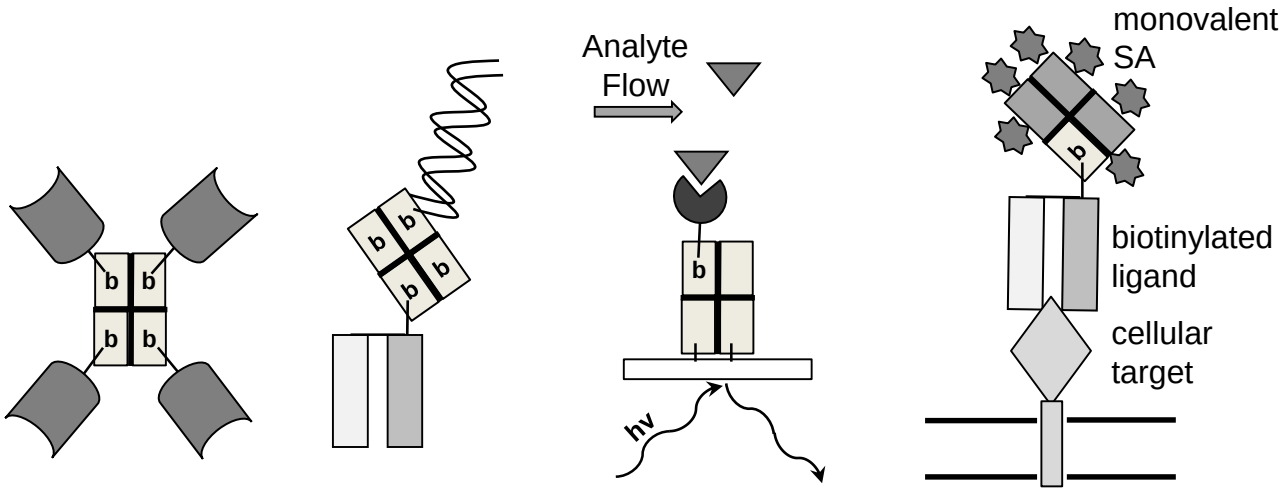
Diverse biotinylated forms, some with impaired binding

Fab-AviTag:

↓ *BirA*

Single biotinylated form, biotin distant from binding site

Figure 2



Tetramerization **Bridging** **Immobilization** **Sensitive detection**

Figure 3

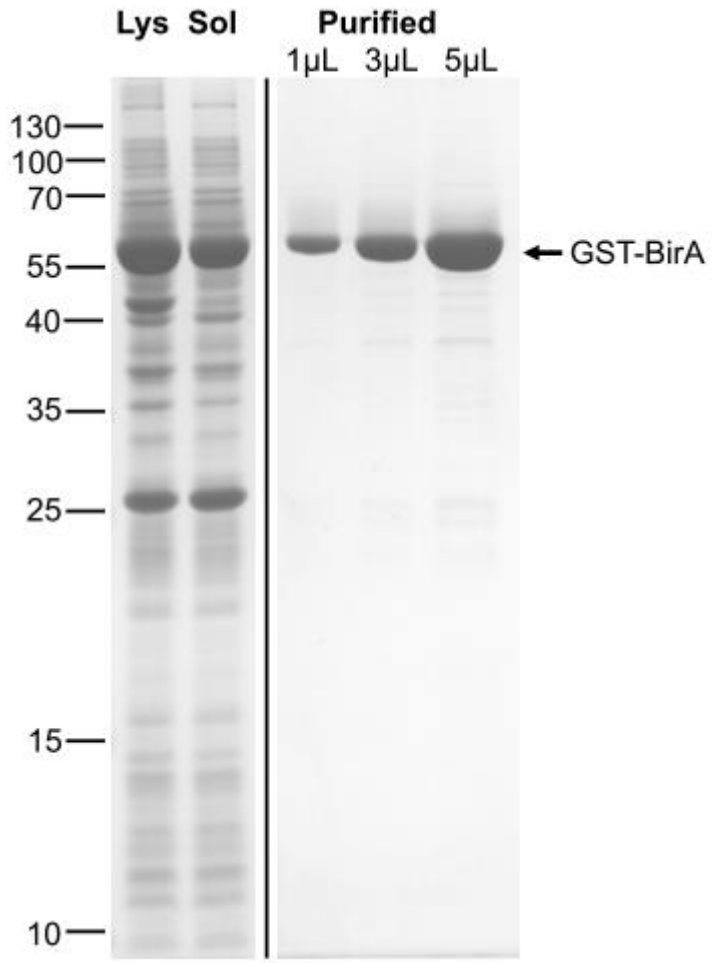

Lys
Sol
Purified
1µL
3µL
5µL
130
100
70
55
40
35
25
15
10
GST-BirA

Figure 4

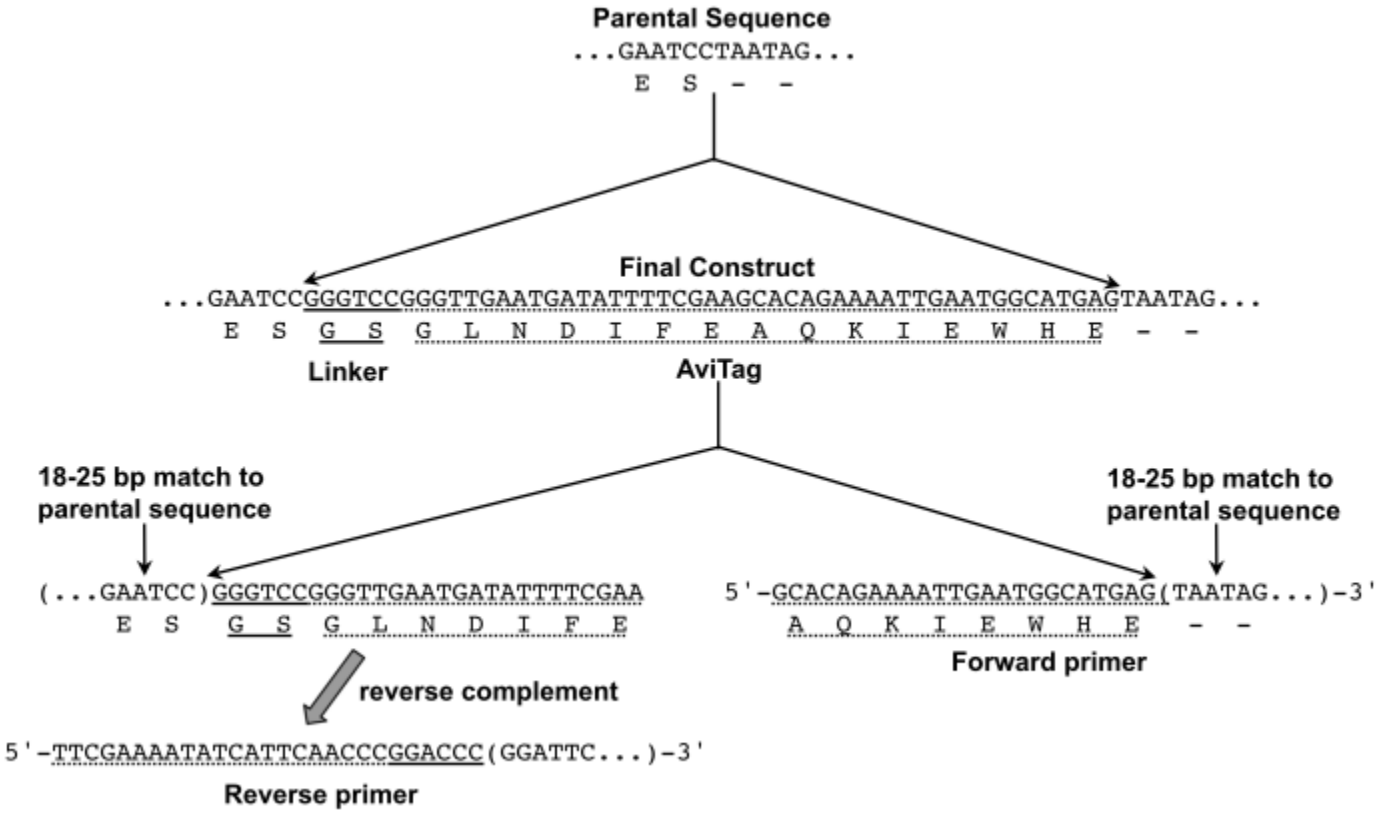
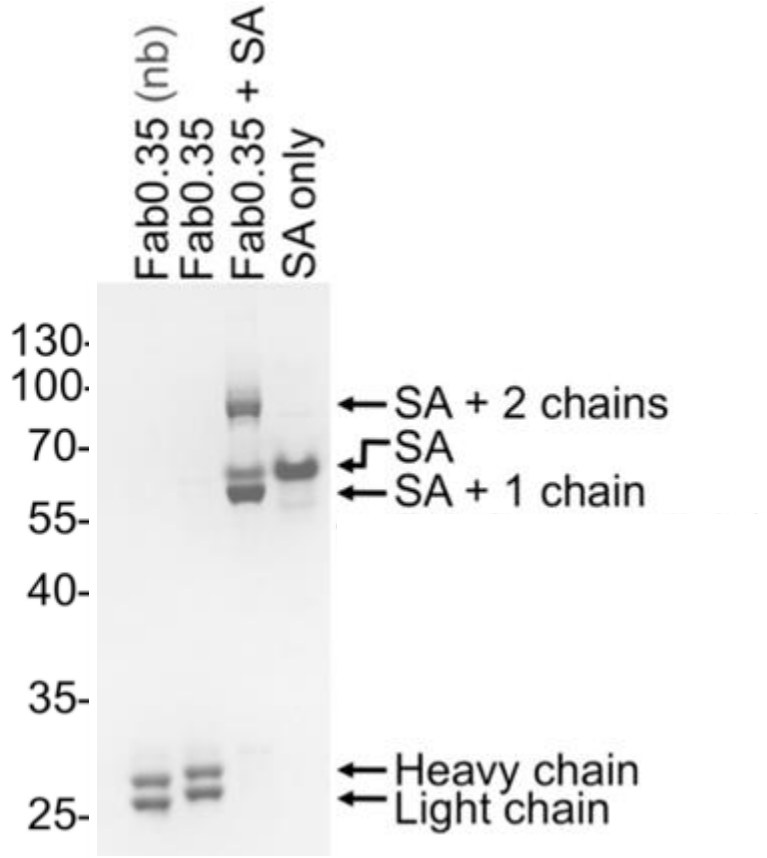

Parental Sequence
...GAATCCTAATAG...
E S - -
Final Construct
...GAATCCGGGTCCGGGTTGAATGATATTTTCGAAGCACAGAAAATTGAATGGCATGAGTAATAG...
E S G S G L N D I F E A Q K I E W H E - -
Linker
AviTag
18-25 bp match to parental sequence
18-25 bp match to parental sequence
(...GAATCC)GGGTCCGGGTTGAATGATATTTTCGAA
E S G S G L N D I F E
5'-GCACAGAAAATTGAATGGCATGAG(TAATAG...)-3'
A Q K I E W H E - -
Forward primer
reverse complement
5'-TTCGAAAATATCATTCAACCCGGACCC(GGATTC...)-3'
Reverse primer

Figure 5


Fab0.35 (nb)
Fab0.35
Fab0.35 + SA
SA only
130
100
70
55
40
35
25
SA + 2 chains
SA
SA + 1 chain
Heavy chain
Light chain