Chapter 14

A Biotin Ligase-Based Assay for the Quantification of the Cytosolic Delivery of Therapeutic Proteins

Wouter P.R. Verdurmen, Marigona Mazlami, and Andreas Plückthun

Abstract

The efficient delivery of external proteins from the external milieu to the cytosol of mammalian cells has great potential for both scientific investigations and future therapies. However, when assessing the cellular uptake of proteins, it is often difficult to distinguish between proteins that are stuck in the endosomes and those that have escaped into the cytosol. Here, we describe a method employing the prokaryotic enzyme biotin ligase that overcomes this problem and which can be employed for a highly sensitive quantification of cytosolic protein delivery.

Key words Biotin, Biotin ligase, Cellular internalization, Cytosolic protein delivery, Streptavidin, Western blot, Protein engineering

1 Introduction

Over the years, many different techniques have been proposed for the quantification of protein delivery into cells. The most popular ones rely either on fluorescence or biological readouts, which, however, each have their own serious drawbacks [1–4]. Fluorescence-based methods typically cannot distinguish well between fluorescence signals from the endosomes and fluorescence signals from the cytosol. Furthermore, cellular fluorescence is difficult to reliably quantify [5, 6]. Additionally, great care must be taken to prevent that the fluorescence microscopy itself might be skewing the result, either through the handling of the cells, especially fixation, or through contributing effects of the fluorophore to endosomal escape, chemically or photochemically. Biological readouts, if carefully controlled, can give direct information about cytosolic delivery, but are generally not quantitative either and require the delivery of an intact "assay" protein such as cre-recombinase [4] or ubiquitin [1]. For the former, only one active molecule can be sufficient and carry out one turnover to be detectable.

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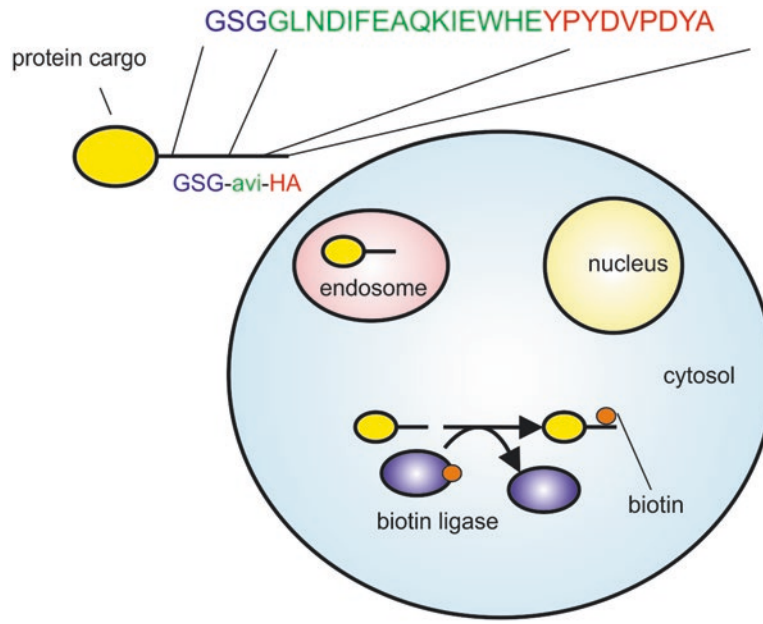

Fig. 1 Principle of the biotin ligase assay. The avi tag fused to a cargo protein gets biotinylated exclusively in the cytosol via an overexpressed bacterial biotin ligase. The biotinylated fraction of the cargo protein represents the fraction that has reached the cytosol. Additionally, the HA tag on the cargo protein remains unchanged during the internalization process and its presence in cell lysates thus reflects total cellular internalization (endosomes or cytosol)

To obtain reliable quantitative information on cytosolic protein delivery, we developed an assay that relies on the cytosolic overexpression of the prokaryotic enzyme biotin ligase and the fusion of a short peptide tag to a cargo protein (Fig. 1) [7]. While all cells express biotin ligases, only the bacterial enzyme BirA biotinylates a peptide of 15 amino acids, referred to as the avi tag, with a very high efficiency, and has no known mammalian targets [8, 9], and conversely, the avi tag is not a substrate of the mammalian enzymes. The assay is applicable to *any* protein and functions by incubating cargo proteins with cells that stably overexpress biotin ligase in the cytosol. The assay thus measures whether the substrate has reached the enzyme.

After the desired incubation time, cells are lysed under harsh conditions immediately, thus avoiding the potential occurrence of biotinylation events after lysis. Lysates are then analyzed by western blotting and probed for the presence of a cargo protein modified with biotin, using fluorescently labeled streptavidin. In parallel, the lysates are probed for the presence of an HA tag, also fused to the cargo protein, which does not get modified during or after transport and thus reflects the total amount of internalized protein. (We refer to "internalized" as the sum of protein in endosomes and the cytosol; in contradistinction to "cytosolic".)

Important controls that are performed in every single assay ensure that the biotinylation of cytosolically localized proteins is complete and that there is no detectable biotinylation after cell lysis. The biotin and HA-derived bands of the protein of interest on the western blots thus separately identify the cytosolic fraction and the total internalized fraction (Fig. 2). The method is highly sensitive, as low nanomolar cytosolic concentrations of delivered proteins can still be reliably quantitated.

Three key features enable the highly sensitive absolute quantitation that can be achieved: first, the exceptionally strong affinity between biotin and streptavidin, reported to be 10^{-15} M [10, 11]; second, the broad linear correlation between the amount of protein present and the signal intensity when using a fluorescence scanner [7], in contrast to detection systems that rely on an enzymatic signal amplification, which are generally less suitable for absolute quantification purposes; third, the SDS-PAGE-based separation of the biotinylated protein of interest from the very low

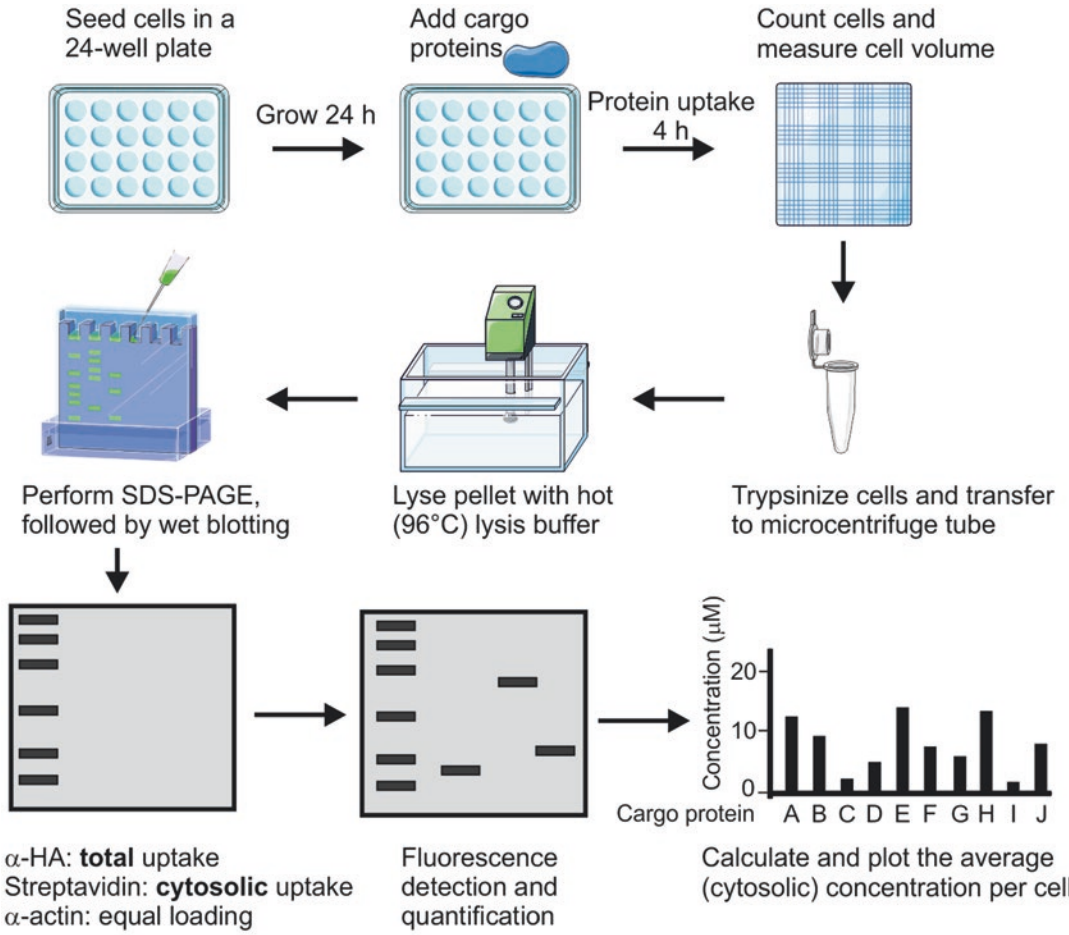

Fig. 2 Schematic representation of the most important steps of the biotin ligase assay

levels of endogenous biotinylated proteins. Altogether, the assay provides a robust way to gain a quantitative insight into the cytosolic delivery of any protein of interest.

2 Materials

All solutions that will be used for live cell culture should be sterile or sterilized before use. Solutions that are used after lysing the cells do not have to be sterile per se, but we highly recommend working with filtered buffers, as unseen particles may cause unwanted background problems in western blotting.

2.1 Cell Culture Reagents

1. Flp-In 293 cell line stably overexpressing biotin ligase (*see* **Note 1**).
2. D-Biotin. To prepare a D-Biotin stock, dissolve D-Biotin to a concentration of 4 mM in water (*see* **Note 2**). Sterile-filter the D-Biotin in a flow-hood. Prepare aliquots of 1 mL and store at −20 °C until further use. Do not reuse aliquots.
3. MG-132 (Calbiochem) (*see* **Note 3**), a proteasome inhibitor. To prepare an MG-132 stock, dissolve MG-132 to a concentration of 42 mM in DMSO. Prepare aliquots of 50 μL. Store at −20 °C until further use. The stock solution can be thawed and frozen several times without loss of activity.
4. Complete medium, Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% Fetal Calf Serum (Amimed), 100 units penicillin and 100 μg/mL streptomycin (100× stock solution from Sigma-Aldrich).
5. Dulbecco's phosphate buffered saline (Sigma-Aldrich).
6. 0.5 mg/mL trypsin solution with 0.2 mg/mL EDTA (Sigma-Aldrich).
7. Transfection reagent TransIT 293 (Mirus) (*see* **Note 4**).
8. Opti-MEM medium (Gibco).
9. pcDNA5/FRT plasmid overexpressing the avi- and HA-tagged unselected designed ankyrin repeat protein (DARPin) E3_5 [12] (*see* **Note 5**).
10. Avi-tagged, non-biotinylated test protein: The protein of which the cytosolic delivery efficiency will be assessed (*see* **Note 6**).
11. Sterile hood for conducting all preparation steps.
12. Sterile incubator set at 37 °C containing 5% CO_2 in the atmosphere.

2.2 Lysis and Western Blotting Components

1. Stabilized avi tag peptide: (Ac-GGLNDIFEAQKIEWHED–NH_2). Purchased from EMC Microcollections. The peptide is N-terminally acetylated and C-terminally amidated to protect

against exoproteolytic degradation. It contains an additional amino acid at either terminus in addition to the 15 amino acids that make up the avi tag. The peptide is to be used as a competitive substrate. Prepare a 10 mM stock in DMSO.

2. Sodium dodecyl sulfate (SDS) lysis buffer: 100 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 10% (v/v) glycerol, 175 mM β-mercaptoethanol, and 0.02% (w/v) bromophenol blue.
3. Digitonin-based extraction buffer: 25, 50 or 100 μg/mL digitonin, 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1 μM leupeptin, and 1.4 μM pepstatin-A.
4. A heating block that can reach temperatures of up to 96 °C.
5. 5× SDS-PAGE sample buffer: 175 mM Tris–HCl, pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 286 mM β-mercaptoethanol, 0.15% (w/v) bromophenol blue.
6. Non-denaturing lysis buffer: 0.5 M Tris–HCl, pH 6.8, 20% (v/v) glycerol, 0.02% (w/v) bromophenol blue.
7. Streptavidin (Promega). Dissolve to a concentration of 1 mg/mL in PBS. Shock-freeze in liquid nitrogen and store aliquots at −20 °C. Thaw aliquots only once and use on the same day.
8. Ammonium persulfate (APS) (Amresco). Prepare a 10% (w/v) solution in water. Store aliquots at −20 °C. A working aliquot may be kept at 4 °C for several months.
9. *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED) (Sigma-Aldrich).
10. Resolving gel 4× stock solution: 1.5 M Tris–HCl, pH 8.8. Store at 4 °C for up to 6 months.
11. Stacking gel 4× stock solution: 0.5 M Tris–HCl, pH 6.8. Store at 4 °C for up to 6 months.
12. Acrylamide solution: Rotiphorese® Gel 30 (37.5:1): 30% acrylamide/bisacrylamide, mixing ratio 37.5:1 (Roth).
13. SDS-PAGE running buffer: 25 mM Tris–HCl, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3.
14. PageRuler: Prestained protein ladder (Thermo Scientific).
15. Western blot transfer buffer: 25 mM Tris–HCl, 0.192 M glycine, 20% (v/v) methanol, pH 8.3 (*see* **Note 7**).
16. Blotting paper (Macherey-Nagel).
17. Immobilon-FL PDVF membrane (Millipore) (*see* **Note 8**).
18. 10× casein blocking buffer (Sigma-Aldrich). Prepare 15 mL aliquots and store them at −20 °C. Thaw only once and use within several weeks (*see* **Note 9**).
19. Phosphate-buffered saline (PBS): 137 mM NaCl, 12 mM phosphate, 2.7 mM KCl, pH 7.4. To make 1 L of 10× PBS, combine 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g

KH_2PO_4. Dilute tenfold to make 1× PBS. The pH should be 6.8 before dilution, and close to 7.4 after dilution. Adjust if necessary.

20. PBS-T: PBS supplemented with 0.1% (v/v) Tween-20.
21. Antibody incubation buffer: PBS-T + 1× casein blocking buffer.
22. 10% (w/v) SDS. Dissolve 5 g SDS in 50 mL. Mix slowly to avoid foam formation. Store at room temperature up to 6 months before discarding.
23. Streptavidin incubation buffer: PBS-T + 1× casein blocking buffer + 0.1% (w/v) SDS.
24. Mini-PROTEAN® Tetra system.
25. Mini-PROTEAN® Tetra cell casting module.
26. Mini-PROTEAN® short plates and spacer plates.
27. 15-well 1 mm combs.

2.3 Antibodies and Detection Reagents

1. Streptavidin IRDye 680LT (LI-COR Biosciences).
2. Polyclonal anti-HA antibody (H6908, Sigma-Aldrich).
3. Mouse anti-actin (ab8224, Abcam).
4. Goat anti-rabbit Alexa Fluor 680 (Invitrogen).
5. Donkey anti-mouse IRDye 800 (Rockland Immunochemicals Inc.).
6. Goat anti-rabbit IRDye 800CW (LI-COR Biosciences).
7. Odyssey IR scanner (LI-COR Biosciences).

3 Methods

Until the point of cell lysis, all procedures should be performed with sterile materials in a sterile flow hood.

3.1 Incubation of Cells with Proteins

1. Seed 300,000 Flp-In 293 cells stably overexpressing biotin ligase in a 24-well plate (*see* **Note 1**). The cells will have a confluence of ~60–80% 24 h after seeding. Use 400 µL complete medium per well for seeding the cells.
2. 24 h after seeding, transiently transfect the cells from a single well on the 24-well plate with a plasmid containing the DARPin HA_E3_5_avi, which carries both an HA- and an avi-tag (*see* **Note 10**). For transfection, combine 1.5 µL TransIT 293 with 500 ng sterile-filtered plasmid DNA in 50 µL Opti-MEM medium. Incubate at room temperature for 30 min to allow complex formation. Add transfection mixture dropwise to the well. Do not exchange the medium in the well before or after the addition of the transfection mixture.

3. Prepare incubation medium: add 0.1 mM sterile-filtered D-Biotin from a 4 mM D-Biotin stock solution to the complete medium (*see* **Note 11**). Then, add the proteasome inhibitor MG-132 to a final concentration of 50 μM, 840× diluted from a 42 mM stock (*see* **Note 12**).
4. 48 h after seeding, incubate cells with cargo proteins to be tested for transport that contain both an avi tag and an HA tag (*see* **Note 13**). Dilute the protein before addition in incubation medium to the desired final concentration, reaching a total volume of 450 μL. Mix well. Fully exchange the medium in the wells with 400 μL of the incubation medium containing the cargo proteins of interest. As soon as the medium has been exchanged, place the 24-well plate back in the incubator at 37 °C for 4 h (*see* **Note 14**).

3.2 Cell Lysate Preparation

1. Add 20 μM of the stabilized avi tag peptide to the SDS lysis buffer (dilute 500-fold from a 10 mM stock). The stabilized avi tag peptide serves as a competitive biotin ligase substrate, such that if any ligase was not quenched completely after lysis with hot SDS-containing buffer, it would mostly work on the synthetic peptide substrate (*see* **Note 15** and Fig. 3). Preheat the peptide-containing SDS lysis buffer at 96 °C. Make sure the lid is tightly closed to avoid buffer evaporation. 50 μL SDS lysis buffer for each condition in a 24-well plate is needed. Prepare at least 20% more than what is strictly needed to cover the possibility of some minor evaporation.
2. After a 4 h incubation time, remove the 24-well plate from the incubator and wash the cells once with 1 mL PBS to remove the protein that is still in the medium.
3. Trypsinize the cells with 100 μL of a 0.5 mg/mL trypsin solution with 0.2 mg/mL EDTA for approximately 5 min, until the cells detach from the surface.
4. Add 800 μL fresh complete medium to dilute the trypsin, mix well, and transfer the contents of the well to a microcentrifuge tube.
5. Centrifuge the cells for 3 min at 300 × *g*.
6. Carefully remove the supernatant with a glass pipette and a suction system. Resuspend the cell pellet in 1 mL PBS. Centrifuge again.
7. Carefully remove the supernatant fully. Be careful not to disturb the pellet. You can now transfer the pellets to a non-sterile working area. Lyse the cell pellets in 50 μL preheated (96 °C) SDS lysis buffer containing the avi tag peptide. Heat the lysates *immediately* for 8 min at 96 °C to prevent biotin ligase from biotinylating avi-tagged proteins after lysis (*see* **Note 16**). Store at −20 °C until further use.

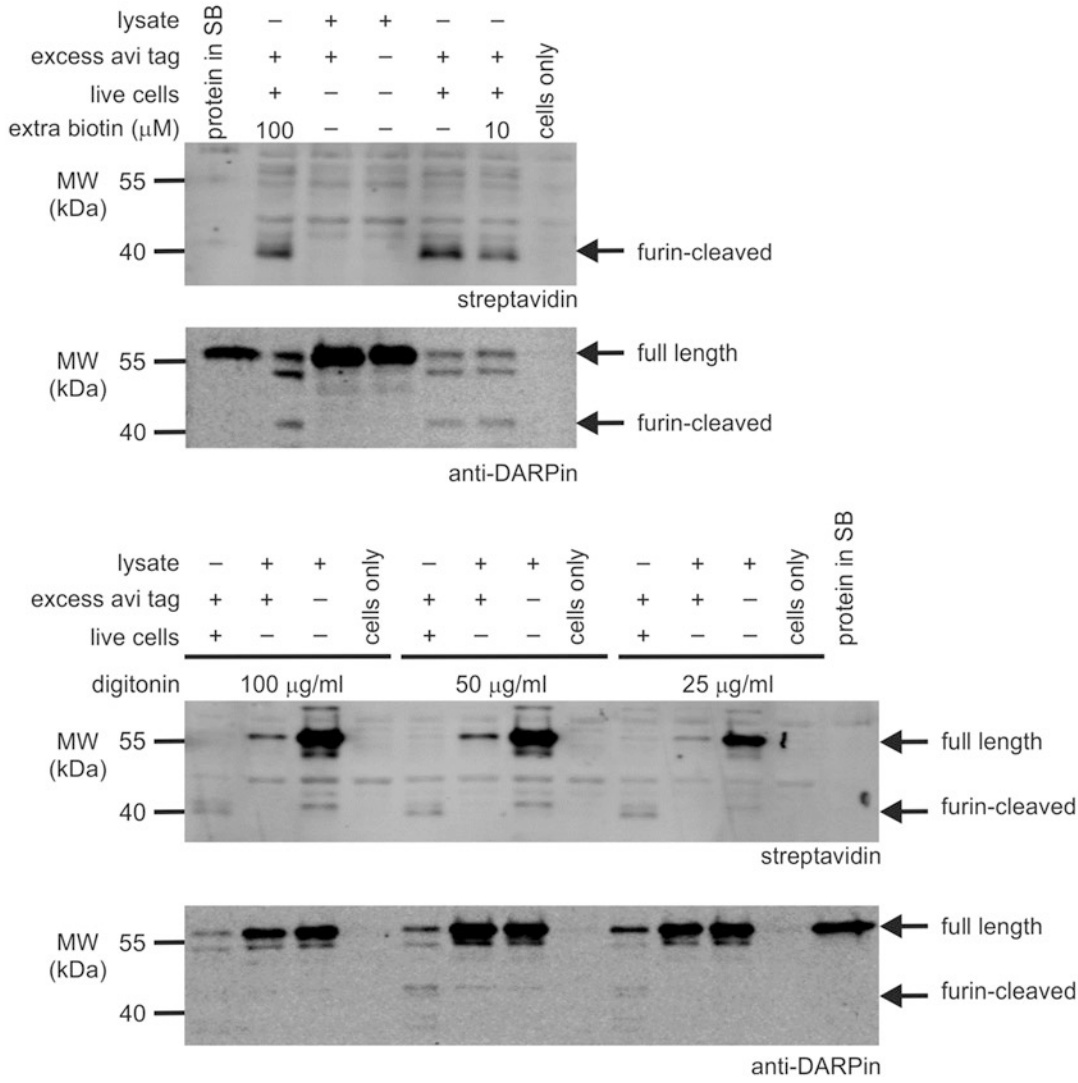

Fig. 3 The effect of adding D-Biotin and the avi tag peptide. The cargo protein Ec1-ETA(252-412)-NI_2C (full length; MW = 55 kDa), which reaches the cytosol only after being cleaved by furin (furin-cleaved; MW = 32 kDa) [7], was used as a model protein to study the role of the avi tag peptide and D-Biotin in the biotin ligase assay. The uncleaved fraction does not reach the cytosol and serves as a convenient internal control for any labeling by BirA after cell lysis. External unbiotinylated ETA(252-412)-NI_2C was either added to biotin ligase-expressing cells (Flp-In 293) at the moment of lysis (labeled "*lysate*") or incubated for 4 h with live cells before lysis (labeled "*live cells*"). In the upper panel, the SDS lysis buffer was used with or without added excess avi tag peptide (20 μM). Since there is no difference between the *lysate* lanes, biotin ligase seems to be completely deactivated in the lysis procedure, and the avi tag peptide is only an additional precaution. Live cells incubated with varying concentrations of extra D-Biotin in the DMEM medium did not produce more furin-cleaved streptavidin-stained band. In the lower panel, lysis was accomplished with a digitonin-based cytosol extraction buffer containing varying amounts of digitonin and also with or without excess avi tag peptide. The full-length protein is strongly labeled, indicating that this procedure does not quench the biotin ligase activity, but addition of avi tag peptide partially inhibits appearance of this band. Note that, even with the avi tag peptide present, the false-positive band of the uncleaved protein is still very intense compared to the *correct* live-cell band for the furin-cleaved protein, underscoring the necessity of using a lysis protocol that instantly abolishes biotin ligase activity (which the digitonin-based buffer evidently does not do). For clarity, the contrast of the blots has been individually adjusted. *Streptavidin*, detection via streptavidin LT680; *anti-DARPin*, detection via polyclonal anti-DARPin serum). *SB*, sample buffer

8. To check for successful immediate abolishing of biotin ligase activity, mix a purified avi-tagged protein with hot (96 °C) SDS lysis buffer containing the stabilized avi tag peptide. Mix briefly and add the hot mixture immediately to a pellet containing untreated biotin ligase-expressing cells from the same experiment. Resuspend well and heat directly for 8 min at 96 °C. Store at −20 °C until further use (*see* **Note 17**).
9. To check for complete biotinylation of cytosolically localized proteins, dilute the lysate from the cells expressing the avi-tagged protein HA_E3_5_avi fourfold with a non-denaturing, nonreducing lysis buffer. Incubate the diluted lysates for 2 h at 4 °C in the presence or absence of (unlabeled) streptavidin (final streptavidin concentration: 50 μg/mL, diluted from a 1 mg/mL stock in PBS; *see* **Note 18**).

3.3 SDS-PAGE and Western Blotting

1. Pour two 12% (w/v) resolving acrylamide gels with a 4% (w/v) stacking acrylamide gel. Use the Mini-PROTEAN® Tetra cell casting module and compatible short plates and spacer plates according to the manufacturer's instructions. Insert a 15-well comb in the stacking gels.
2. Load 12 μL of each cell lysate sample in the slots. Also include: (a) 1.5 μL of a prestained protein MW marker; (b) 6 μL of a 84 nM solution of a fully biotinylated test protein diluted in SDS lysis buffer that contains both the avi tag and an HA tag; (c) untreated biotin ligase-expressing cells; (d) the diluted lysates of cells expressing HA_E3_5_avi, pre-incubated in the absence or presence of streptavidin (see the previous section). Repeat the procedure for the second gel, thus generating two identically loaded gels.
3. Run the gels in a Mini-PROTEAN® Tetra system at 50 V until the samples enter the resolving gel. Increase the voltage to 150 V and run the gel for an additional 1–1.5 h until the dye front reaches the bottom of the resolving gel.
4. While the gels are running, cut PVDF-FL membranes to match the size of the gel (70 × 85 mm). Activate the membranes with methanol, wash them with ultrapure water, and keep them in transfer buffer until use.
5. When the dye front has reached the bottom of the gels, separate the gel plates, remove the stacking gels, and put the resolving gels in transfer buffer.
6. Prepare the blotting sandwich as follows. Place an absorbent membrane in the holder and add 3 Whatman no. 3 filter papers (80 × 90 mm). Add the blotting membranes. Place the gels carefully on the top of the membrane. Avoid moving the gels afterward, as this may cause smearing. Add three additional Whatman no. 3 filter papers, place a second absorbent membrane on top and close the blotting sandwich with a clamp.

Insert the blotting sandwich in the Mini-PROTEAN® Tetra system for wet blotting.

7. Run the protein transfer with a constant voltage of 100 V for 1 h.
8. Remove the blotting sandwich and inspect the blots. The protein marker should be visible on the PVDF-FL membranes, which is a first indication for successful transfer.
9. Place the membranes in 1× casein blocking buffer and block for 20 min.
10. Incubate one of the membranes for 1 h at ambient temperature with streptavidin IRDye 680LT (1:10,000; LI-COR Biosciences) in streptavidin incubation buffer (contains 0.1% (w/v) SDS). In parallel, incubate the other membrane for 1 h in antibody incubation buffer, which does not contain SDS, with a 1:1,000 dilution of the rabbit anti-HA antibody and a 1:5,000 dilution of the mouse anti-actin antibody (*see* **Note 19**).
11. After the incubation with the primary antibody, wash the membranes 4 × 5 min with PBS-T.
12. Incubation with a secondary antibody is only required for the membrane incubated with the anti-HA and anti-actin antibodies. Use a goat anti-rabbit Alexa Fluor 680 antibody for detecting the anti-HA antibody and a donkey anti-mouse IRDye 800 antibody for detecting the anti-actin antibody. A 1:5,000 dilution in antibody incubation buffer is suitable for both secondary antibodies. Incubate for 45 min at ambient temperature.
13. After the incubation with the secondary antibody, wash the membranes 4 × 5 min with PBS-T.
14. Fluorescent signals can be acquired with an Odyssey IR scanner (LI-COR Biosciences).
15. Use the Image Studio Lite (LI-COR) software to quantify band intensities.
16. On the basis of knowing the number of cells in the lysate, a measurement of the average cell volume, e.g., by the Coulter principle [13], an estimate of the volume fraction of the cytosol (related to the total volume of the cell, assumed to be approximately one), and the band intensities of the directly loaded biotinylated and HA-tagged control protein, it is straightforward to calculate back the amount of protein delivered into the cytosol for each of the test conditions.

4 Notes

1. We have the most experience with an Flp-In 293 cell line stably overexpressing BirA and EpCAM, but the method is in no way restricted to or adapted to this cell line. Experiments

in at least four other BirA-overexpressing cell lines have demonstrated that the presented method works well in all of them. Stable cell lines can be generated from single clones as well as from pools that are under the constant pressure of a selection agent [14].

2. D-Biotin is poorly soluble at low pH, but becomes soluble in dilute alkali (pH ~10). Mild heating (below 50 °C) also aids in the solubilization of D-Biotin. Make sure the pH of the D-Biotin solution is very carefully titrated, as D-Biotin is less stable at very basic pH values. Also the pH of the cell culture medium must be checked after the addition of the D-Biotin solution, since the base used for dissolving D-Biotin may shift the pH.
3. We chose MG-132 because it is a well-established, affordable proteasome inhibitor. Other proteasome inhibitors may work just as well. 50 μM MG-132 will be sufficient for most, if not all, cell lines. Lower concentrations can likely be used for certain cell lines, but the efficiency would have to be tested in each specific case.
4. For Flp-In 293 cells, we have good experiences with the TransIT 293 reagent from Mirus. Generally speaking, any transfection reagent suitable for the cell line being investigated will be appropriate for this step.
5. The cytosolic overexpression of an avi-tagged protein serves as a control showing that a cytosolically localized protein becomes fully biotinylated quickly after expression. Although we use HA-E3_5-avi for this purpose, any protein with both an HA and an avi tag will be suitable for this purpose (*see* **Note 10**).
6. There are many ways to produce a protein with an avi tag attached to it. Our standard approach is a genetic fusion that is overexpressed in *E. coli*. With this approach, care must be taken to remove the fraction that has already been biotinylated in *E. coli*. The biotin ligase gene is essential for survival [15], so this gene cannot be removed in a dedicated expression strain. The removal of the biotinylated fraction of the overexpressed protein can be accomplished with a commercially available streptavidin resin using the manufacturer's protocol. We have good experiences with the streptavidin resin from Genscript.
7. Western blot transfer buffer can be easily prepared from a tenfold stock solution (250 mM Tris–HCl; 1.92 M glycine) without methanol. Adjusting the pH should not be necessary, although checking the final pH is of course recommended, which should be 8.3.
8. Using a membrane that has been specifically fabricated for the use with fluorescent reagents is strongly recommended. In our experience, other membranes have given high background levels and thus resulted in a greatly reduced sensitivity.

9. Milk should be avoided when using detecting streptavidin–biotin interactions. Endogenous biotin present in milk will cause a high background signal [16].
10. To ensure that all the cells in the experiment express sufficient amounts of biotin ligase for the full biotinylation of a delivered cargo protein, we include in all experiments one well in which we overexpress a protein in the cytosol, which is both HA- and avi-tagged. The cytosolic localization of the entire fraction of the protein means that it should be 100% biotinylated. To check this, cell lysates of BirA-overexpressing cells in which this avi-tagged protein was overexpressed are divided into two aliquots after lysis. One aliquot is pre-incubated with streptavidin before SDS-PAGE, whereas the other is not. Since the streptavidin–biotin incubation survives SDS-PAGE, the full biotinylation of the overexpressed protein can be checked by a *complete* shift of the band representing the avi-tagged protein (i.e., from the size of the "unshifted" avi-tagged protein to the size of the "shifted" avi-tagged protein-streptavidin complex).
11. Although we add 0.1 mM D-Biotin in all our assays, we have not observed an evident reduction in biotinylation efficiency of delivered proteins when additional D-Biotin was omitted (Fig. 3). Nevertheless, it is still possible that in some instances the omission of extra D-Biotin might reduce the biotinylation efficiency, thus potentially underestimating the rate or amount of delivered protein. For this reason, we prefer to always include it, as we have not observed any negative effects of D-Biotin addition.
12. The amount of intact biotinylated protein that is present in the cytosol at any one time is a function of the rate of delivery and the rate of degradation. To minimize the rate of degradation in 4-h uptake experiments, we add the proteasome inhibitor MG-132. In a 4-h uptake experiment, proteasomal degradation is generally the main mechanism of protein breakdown in the cytosol. For longer incubations, the role of autophagy becomes more important. It is important to note that proteasome inhibition will induce apoptosis after extended times (e.g., 20 h) due to the aberrant accumulation of ubiquinated proteins.
13. The specific concentrations required for the incubation are not part of this protocol as they are a function of the protein that is to be delivered and of the receptor that is being targeted. It is to be advised to test a broad concentration range, if only few proteins are being investigated. If many proteins are being investigated, we would recommended that for proteins that bind a receptor with a high affinity (K_D < 10 nM), 200 nM is first examined, whereas for proteins that have a receptor-independent mechanism of uptake, a concentration of 1 μM can be used as a starting concentration.

14. The incubation time can of course be varied. The desirable time depends on many factors, including the nature of the protein transporter being investigated, the cell line in question and whether the rate of internalization or the total accumulation will be investigated.
15. The addition of an excess of a non-biotinylated stabilized avi tag peptide serves to quench any possible residual activity of biotin ligase. Although we have not seen a residual activity with direct lysis using a hot 4% (w/v) SDS-containing buffer, this activity can be seen with milder forms of lysis, such as using a digitonin-based cytosol extraction buffer (Fig. 3). Nevertheless, it is difficult to exclude a very low (i.e., undetectable) level of residual biotin ligase activity. For this reason, we include an excess of avi tag peptide as a competitive substrate in the SDS lysis buffer in all experiments as a precaution.
16. In order to prevent false-positive biotinylation events of avi-tagged proteins that occur after lysis, it is absolutely essential that biotin ligase activity is fully abolished at the moment of lysis (*see* **Note 15**).
17. The complete absence of biotinylation of the avi-tagged protein, as determined by western blotting, indicates that all biotin ligase activity had been stopped immediately at the moment of lysis.
18. To confirm biotinylation activity of biotin ligase overexpressing cells, the mixtures from this step are investigated for a complete band-shift via western blot of the transfected protein (avi tag and antibody-detectable tag should be at opposite termini to avoid interference). A complete shift confirms the activity of biotin ligase in the cells of choice (*see* **Notes 5** and **10**).
19. Streptavidin IRDye 680LT incubations should be performed in the presence of 0.1% (w/v) SDS to reduce the nonspecific signal intensity. In contrast, the antibody incubations should be performed in the absence of SDS, as this may decrease the signal intensities. Since three signals will be obtained from each cell lysate (e.g., from biotin, HA, and actin), it is better to combine the anti-HA and anti-actin antibodies on the same blot than combining one of the antibodies with streptavidin IRDye 680LT on the same blot, as the antibodies require similar incubation conditions.

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