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Bacterial peptide display for the selection of novel biotinylating enzymes

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Dear Dr. Vineeta Bajaj,

Thank you for your kind email of June 14 2019 with the possibility to revise our manuscript (JoVE60266)

We are pleased to re-submit the revised manuscript with the suggested changes and we have addressed the reviewers' concerns as outlined in the attached response letter.

We look forward to hearing from you.

Sincerely,
Per Svenningsen, PhD
Associate Professor
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TITLE:

Bacterial Peptide Display for the Selection of Novel Biotinylating Enzymes

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Directed evolution, random mutagenesis, labeling, protein engineering, protein-biotin ligase, protein tag

SUMMARY:

Here we present a method to select for novel variants of the *E. coli* biotin-protein ligase BirA that biotinylates a specific target peptide. The protocol describes the construction of a plasmid for the bacterial display of the target peptide, generation of a BirA library, selection and characterization of BirA variants.

ABSTRACT:

Biotin is an attractive post-translational modification of proteins that provides a powerful tag for the isolation and detection of protein. Enzymatic biotinylation by the *E. coli* biotin-protein ligase BirA is highly specific and allows for the biotinylation of target proteins in their native environment; however, the current usage of BirA mediated biotinylation requires the presence of a synthetic acceptor peptide (AP) in the target protein. Therefore, its application is limited to proteins that have been engineered to contain the AP. The purpose of the present protocol is to use the bacterial display of a peptide derived from an unmodified target protein to select for BirA variants that biotinylates the peptide. The system is based on a single plasmid that allows for the co-expression of BirA variants along with a scaffold for the peptide display on the bacterial surface. The protocol describes a detailed procedure for the incorporation of the target peptide into the display scaffold, creation of the BirA library, selection of active BirA variants and initial characterization of the isolated BirA variants. The method provides a highly effective selection system for the isolation of novel BirA variants that can be used for the further directed evolution of biotin-protein ligases that biotinylate a native protein in complex solutions.

INTRODUCTION:

Biotinylation of a protein creates a powerful tag for its affinity isolation and detection. Enzymatic protein biotinylation is a highly specific post-translational modification catalyzed by biotin-protein ligases. The *E. coli* biotin-protein ligase BirA is extremely specific and covalently biotinylates only a restricted number of naturally occurring proteins at specific lysine residues[1]. The advantages of the BirA catalyzed biotinylation are currently harnessed by fusing the target protein with a small synthetic 15-amino-acid biotin acceptor peptide (AP) that is effectively biotinylated[2] and allows for the highly specific and efficient in vivo and in vitro biotinylation by co-expression or addition of BirA[3-5]. Although the in vivo and in vitro BirA catalyzed biotin-protein ligation is an attractive labeling strategy, its application is limited to samples that contain AP-fused proteins. The purpose of this method is the development of new mutants of biotin-protein ligases that selectively biotinylate native unmodified proteins and, thereby expand the number of applications in which the enzymatic biotinylation strategy can be used.

Protein function can be evolved through iterative rounds of the gene mutation, selection, and amplification of gene variants with the desired function. A strong and efficient selection strategy is crucial for the directed evolution and biotin-protein ligase activity is readily selected due to the strong binding between biotin and streptavidin and its homologs[6]. Phage display technologies allow for the selection of phages that display biotinylated peptides[7,8]. Since amplification of isolated phages requires infection of a bacterial host, however, the phage selection with streptavidin creates a bottleneck in that the high-affinity binding of biotin to streptavidin is virtually irreversible under non-denaturing conditions. To ensure reversible binding of biotinylated phages, monomeric avidins with lower affinity were used which resulted in a modest ~10-fold enrichment[7]. We recently developed a bacterial display method for the isolation of novel BirA variants that eliminates the need for the elution from the affinity matrix and thereby removes a bottleneck from previous BirA selection systems[9]. Indeed, our bacterial display system allows for a >1,000,000-fold enrichment of active clones in a single selection step[9], thus providing an effective selection system for the directed evolution of novel BirA variants.

Our bacterial display system consists of two components, BirA with a C-terminal 6xHis tag and a scaffold protein that allows for the surface display of a target peptide. We used the scaffold protein enhanced circularly permuted outer membrane protein X (eCPX) since the effective display of peptides can be observed at both the N- and C-termini[10,11]. The fusion of the target peptide sequence to the C-terminus of eCPX ensures biotinylation of bacteria expressing active BirA variants. The bacteria allow for the effective streptavidin selection as the biotinylated peptide now displays on the surface (**Figure 1a**).

The purpose of this method is to select for novel variants of BirA that biotinylates peptide sequences present in native proteins. The system is encoded by genes present on the plasmid pBAD-BirA-eCPX-AP, which contains an arabinose-inducible promoter controlling BirA (araBAD), and a T7 promoter controlling eCPX[9] (**Figure 1b**). The present protocol describes the detailed procedure for 1) incorporation of a peptide derived from a target protein into the C-terminal of eCPX, 2) creation of a mutational library of BirA by error-prone PCR, 3) selection of streptavidin-binding bacteria by magnetic-activated cell sorting (MACS), 4) quantification of bacteria

enrichment, and 5) initial characterization of isolated clones.

PROTOCOL:

1. Insertion of peptide coding sequencing sequence in pBAD BirA-eCPX-AP

NOTE: To select for BirA variants that biotinylate a native target protein, start by identifying a 15-amino acid peptide sequence in the proteins primary sequence that contains at least one lysine (K) residue.

1.1. Go to the sequence manipulation suite[12].

1.2. Paste the identified 15-amino acid peptide sequence into the input box in FASTA format and press **Submit**.

1.3. Select and copy the 45-nucleotide reverse translation of the peptide sequence.

1.4. Download the GenBank File for pBAD-BirA-eCPX-AP from http://n2t.net/addgene:121907.

1.5. Load the file in a plasmid editor (e.g., ApE) and, in the feature window, select the **AP sequence** designated "AviTag(TM)".

1.6. Right-click the highlighted **AP sequence** in the DNA sequence window and select **Paste Rev-Com** in the contextual menu.

NOTE: The coding sequence of eCPX is in the reverse direction and the peptide coding sequence should, therefore, be pasted as a reverse complement.

1.7. Right-click the highlighted sequence and select **New Feature**.

1.8. Add a descriptive name of the inserted sequence and press **OK**.

1.9. Select **Save As** in the **File** menu to save the modified file.

1.10. Design the forward primer to include the last 30 nucleotides of the reverse complement of the peptide coding sequence and add the plasmid binding nucleotide sequence (3'-GCGGCCGCCTGC-5') to its 5' end.

1.11. Design the reverse primer to include the first 30 nucleotides of the reverse complement of the peptide coding sequence and add the plasmid binding nucleotide sequence (3'-CTTAAGTAATGTTTAAACGAATTCGAG-5') to its 5' end.

1.12. Set up a 20 µL PCR reaction in a thin-walled PCR tube by adding the reagents listed in **Table 1.**

1.13. Transfer the tube to a thermal cycler and perform PCR using a program with an initial denaturing at 98 °C for 30 s, 30 cycles of [15 s at 98 °C, 15 s at 60 °C and 3 min at 72 °C], final extension for 2 min at 72 °C and hold at 4 °C.

1.14. Run 5 µL of the PCR reaction on a 1% agarose gel to confirm the amplification of a ~6 kb PCR product.

NOTE: If no PCR product is observed, optimize the PCR condition according to the manufacturer's instructions. The protocol can be paused here.

1.15. Add 1 µL of DpnI to the PCR reaction and incubate for 1 h at 37 °C

1.16. Transform the competent *E. coli* with 2 µL of DpnI digested PCR reaction and plate on Lysogenic broth (LB)/Amp plates. Incubate overnight at 37 °C.

1.17. Inoculate 5 mL LB containing 100 µg/mL ampicillin with a single colony. Incubate overnight at 37 °C with shaking at 200 rpm.

NOTE: It is recommended to test at least 6 colonies.

1.18. Make freeze stocks of 850 µL of each overnight culture by adding glycerol to a final concentration of 15% to the culture. Store at -80 °C.

1.19. Extract the plasmid DNA from the remaining 4 mL culture by mini-prep DNA extraction kit.

1.20. Confirm the correct insert of the peptide coding sequence by DNA sequencing using T7 Terminal primer (GCTAGTTATTGCTCAGCGG).

2. Generation of a BirA library

NOTE: The initial BirA mutational library (**Figure 1c**, step 1) is created by error-prone PCR. Other methods to generate the BirA mutational library are likely to work as well.

2.1. Synthesize the mutant megaprimers with BirA-6xHis forward (ATGAAGGATAACACCGTGCC) and reverse (TCAATGATGATGATGATGATGTTT) primers using 1 ng of pBAD-BirA-eCPX with the target peptide sequence (prepared and confirmed in step 1.20) as a template and 35 PCR cycles with an annealing temperature of 60 °C according to the manufacturer's instruction.

2.2. Run 5 µL of the amplification reaction on a 1% agarose gel to verify the amplification of a 984-bp PCR product.

2.3. Purify the PCR product from the remaining 45 µL of the amplification reaction using a commercial PCR purification kit and use a spectrophotometer to quantitate the DNA yield.

NOTE: Purification from a single 45 µL amplification reaction generally produces a enough yield (>250 ng).

2.4. Prepare the sample reaction in a thin-walled PCR tube by adding the reagents listed in **Table 2**.

2.5. Transfer the reaction mixture to a thermal cycler and run the PCR with the mutant megaprimers prepared in step 2.3 using the following parameters: 1 min at 95 °C and 25 cycles of 50 s at 95 °C, 50 s at 60 °C and 12 min at 68 °C. Store the reaction at 4 °C.

2.6. Add 1 µL of DpnI restriction enzyme directly to the amplification reaction and mix gently.

2.7. Spin down the reaction mixture and incubate for 2 h at 37 °C.

2.8. Transform T7 Express lysY/Iq competent *E. coli* cells with 2 µL of the DpnI reaction.

2.9. Inoculate 100 mL LB containing 100 µg/mL ampicillin with the transformed cells and incubate overnight at 37 °C with shaking at 200 rpm.

2.10. Make freezer stocks with 10 mL of the overnight culture in LB with 15% glycerol and store at -80 °C.

3. Selection of bacteria expressing biotinylated peptide

NOTE: This part of the protocol covers step 2-5 of **Figure 1c**. It is highly recommended that the selection approach is setup using pBAD-BirA-eCPX-AP and pBAD-BirA-eCPX-AP(K10A) as positive and negative controls.

3.1. Inoculate 100 mL of LB containing 1% glucose and 100 µg/mL ampicillin with 1 mL of BirA library and incubate overnight at 37 °C with shaking at 200 rpm.

3.2. Inoculate 5 mL of LB containing 1% glucose and 100 µg/mL ampicillin with 100 µL of the overnight culture.

3.3. Incubate for 2 h and 30 min until the culture reaches an OD_{600} of approximately 0.5.

3.4. Induce eCPX and BirA expression with 0.2% w/v L-arabinose, 100 µM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 100 µM biotin and shake the culture at 200 rpm for 1 h at 37 °C.

3.5. Centrifuge the culture for 10 min at 5,000 *x g* and remove the supernatant.

3.6. Resuspend the cells in 1 mL of the ice-cold PBS and centrifuge at 5,000 x *g* for 5 min.

3.7. Discard the supernatant and resuspend the cells in 400 µL of ice-cold PBS and store cells on the ice.

3.8. Remove 10 µL of resuspended cells and store on the ice in a 1.5 mL tube labeled “Input”.

3.9. Wash 20 µL of streptavidin magnetic beads in 1 mL of ice-cold PBS and place the tube in a bench top magnetic particle separator before carefully removing the supernatant.

3.10. Resuspend the streptavidin magnetic beads in 20 µL of ice-cold PBS and transfer to the 490 µL of resuspended cells from step 3.8.

3.11. Mix by gently pipetting and then incubate for 30 min at 4 °C.

NOTE: Do not vortex as this may lyse the cells. Aggregation of beads is often observed with a high abundance of bacteria displaying a biotinylated peptide.

3.12. Place a column with ferromagnetic spheres in a magnetic particle separator and wash with 5 mL of ice-cold PBS.

3.13. Transfer cells and streptavidin magnetic beads to the column attached in the separator.

3.14. Once the column reservoir is empty, add 500 µL of ice-cold PBS and repeat until the column has been washed with a total volume of 5 mL of ice-cold PBS.

3.15. Remove the column from the separator and place it in a 1.5 mL tube.

3.16. Pipette 1 mL of the ice-cold PBS onto the column and elute the magnetically labeled cells by applying the plunger supplied with the column.

3.17. Transfer the 1.5 mL tube to a bench top separator and wash the magnetically labeled cell with 1 mL of ice-cold PBS.

3.18. Gently resuspend the magnetically labeled cells in 1 mL of the ice-cold PBS before removing and storing 10 µL of the resuspension in a 1.5 mL tube labeled “Output”.

3.19. Inoculate 100 mL of LB containing 1% glucose and 100 µg/mL ampicillin with the magnetically labeled cells and incubate overnight at 37 °C with shaking at 200 rpm.

3.20. The next day, use 10 mL of the overnight culture to make freezer stocks with cells in LB with 15% glycerol and store at -80 °C and 1 mL of the overnight culture for the next round of selection, i.e., step 3.2.

NOTE: Generally, 3-5 rounds of selection are recommended for the enrichment of streptavidin binding bacteria.

4. Quantification of enrichment

NOTE: Quantification of the live bacteria in the "input" and "output" samples are performed after each selection round by plating of serial dilutions of the samples and subsequent counting of colony forming units (CFUs).

4.1. Add 990 µL ice-cold PBS to the input (from step 3.8) and output (from step 3.18) samples and label the tubes "Input 10^{-2}" and "Output 10^{-2}", respectively.

4.2. Make 10-fold serial dilutions of the "Input 10^{-2}" and "Output 10^{-2}" samples in ice-cold PBS until a final dilution of 10^{-10} is reached in the input sample and 10^{-4} in the output sample.

4.3. Plate 100 µL of samples from "Input 10^{-6}", "Input 10^{-8}", "Input 10^{-10}", "Output 10^{-2}", "Output 10^{-3}" and "Output 10^{-4}" on LB/Amp plates and incubate overnight at 37 °C.

4.4. Count the number of colonies on the plates with clearly separated colonies. Multiply the colony count with the dilution factor to obtain the bacterial concentration count/100 µL.

4.5. Calculate the total bacterial count in the input and output samples by multiplying the cell concentration with the input (400 µL) and output volume (1 mL), respectively, and estimate the enrichment by dividing output with input cell count.

NOTE: A significant enrichment should be visible after 3-5 selection rounds

5. Characterization of selected BirA variant

NOTE: The characterization can be performed after selecting BirA variants from the first BirA library; however, the BirA variants generally have low activity towards the peptide. Therefore, an additional round of mutation and selection can also be performed before the characterization. Usually, 10 clones from the final selection round are isolated for further characterization.

5.1. Inoculate 5 mL LB containing 100 µg/mL ampicillin with selected clones from the final selection round. In addition, inoculate 5 mL LB containing 100 µg/mL ampicillin with T7 Express lysY/Iq E. coli transformed with pBAD-BirA-eCPX-AP, which will be used as a positive control for the western blot described below.

5.2. Incubate overnight at 37 °C with shaking at 200 rpm.

5.3. Make freeze stocks of 850 µL of each overnight culture by adding glycerol to a final concentration of 15% to the culture. Store at -80 °C.

5.4. Inoculate 5 mL of LB containing 100 µg/mL ampicillin with 100 µL of overnight culture and incubate at 37 °C with shaking at 200 rpm for 2 h.

5.5. Extract the plasmid DNA from the remaining 4 mL culture by s commercially mini-prep DNA extraction kit. Perform the DNA sequence in forward and reverse directions of the BirA variants with pBAD (ATGCCATAGCATTTTTATCC) and pTrcHis rev (CTTCTGCGTTCTGATTTAATCTG) primers.

5.6. Add 0.2% w/v L-arabinose and 100 µM IPTG and 100 µM biotin to cultures from step 5.4 and shake the culture at 200 rpm for 1 h at 37 °C to induce the expression of eCPX and BirA variants.

5.7. Transfer 65 µL of the culture to 1.5 mL tubes containing 25 µL of the sample loading buffer and 10 µL of the reducing agent.

5.8. Incubate at 95 °C for 5 min.

5.9. Load the sample (including the positive control) onto a 12% SDS-polyacrylamide gel along with a size marker and perform gel electrophoresis at 200 V for approximately 45 min until the 20, 25 and 37 kDa standard bands are clearly separated.

5.10. Release the gel from the cassette and assemble the sandwich for blotting of the gel onto a PVDF membrane.

5.11. Electroblot at 35 V for 2 h on ice.

5.12. Remove the PVDF membrane and incubate in blocking buffer [PBS, 0.05% Tween-20, 3% Skim Milk Powder] for 1 h at room temperature with shaking.

5.13. Prepare the biotin-detection solution by diluting streptavidin-HRP 1:1,000 in PBST.

NOTE: The blocking buffer contains biotin and should therefore not be used as dilution buffer for streptavidin-HRP.

5.14. Discard the blocking buffer, wash the membrane swiftly in PBST [PBS, 0.05% Tween-20] and add the biotin-detection solution. Incubate for 1 h at room temperature with shaking.

5.15. Discard the biotin-detection solution and wash the membrane thoroughly in PBST for 5 min twice and 10 min once with gentle shaking at room temperature.

5.16. Discard the PBST, add 2 mL ECL mix and incubate for 1 min with shaking.

5.17. Dry the membrane swiftly on a tissue paper and develop the image on an X-ray film or in

a digital gel imaging system.

NOTE: In the lane loaded with the positive control, two distinct streptavidin-reaction bands should be clearly visible: a 30 kDa biotinylated endogenously expressed protein and the ~22 kDa eCXP-AP band. If the 10 selected colonies can biotinylate the displayed peptide, a band at ~22 kDa should be visible. The intensity of these bands is generally lower than the positive control and may, therefore, require longer exposure times.

REPRESENTATIVE RESULTS:

Western blot of pBAD-BirA-eCPX-AP expressing bacteria produces a ~22 kDa streptavidin-reacting band consistent with the molecular weight of eCPX (**Figure 2a**). Unlike BirA-6xHis, biotinylated eCPX-AP was present in both uninduced and induced cultures (**Figure 2a**) due to a small degree of T7 promoter activity even in uninduced cultures and subsequent biotinylation of the AP by endogenous BirA. In BirA-eCPX-AP(K10A) expressing cultures, no biotinylated eCPX band was detected (**Figure 2a**). The strong surface biotinylation in the eCPX-AP expressing bacteria causes aggregation upon addition of streptavidin magnetic beads and the formation of a pellet at the bottom of a tube (**Figure 2b**). In the eCPX-AP(K10A) expression bacteria, streptavidin-bead aggregation and precipitation was not observed (**Figure 2b**). Analysis of the precipitate from the streptavidin pulldown, displays a clear 22-kDa streptavidin-reacting and anti-6xHis band in the samples from eCPX-AP cultures, but not eCPX-AP(K10A) cultures (**Figure 2c**). Similarly, the count of bacteria bound to the streptavidin-beads was significantly higher in the eCPX-AP than the eCPX-AP(K10A) cultures (**Figure 2d**).

To select for BirA variants that biotinylate a target peptide, its DNA sequence was incorporated into the C-terminal of eCPX by PCR using the primers designed in step 1.10 and 1.11. An example of the primers designed for the incorporation of a peptide sequence derived from the α-subunit of the epithelial Na^+ channel (ENaC) is shown in **Figure 3a**. After PCR, a 5-µL aliquot was analyzed by agarose gel electrophoresis and a clear and strong band at ~5900 bp was observed (**Figure 3b**).

After the generation of the BirA mutation library, the selection of active BirA variants was initiated. A low degree of streptavidin-bound vs. input bacteria was expected after the first selection round. However, after 2^{nd} and 3^{rd} selection rounds a clear enrichment was observed in the degree of streptavidin-bound bacteria **Figure 4a**). If a clear enrichment is not detected (**Figure 4b**), it is indicative of the failure of the BirA variants to biotinylate the peptide and another peptide sequence should, therefore, be tested.

After the final selection round, 10 clones were characterized by western blotting for their ability to biotinylate the displayed peptide (**Figure 4c**). In the positive control (i.e., AP), ~22 kDa band corresponding to the biotinylated eCPX-AP was observed in a western blot probed with streptavidin-HRP (**Figure 4c**). In the tested clones, a band at similar size was indicative of biotinylation of the displayed peptide fused to eCPX (**Figure 4c**). The intensity of the ~22 kDa bands was lower than the intensity of the eCPX-AP band in the positive control, indicating a lower activity of the isolated BirA variants. The isolated clones can, therefore, be used as a

template for another round of mutations and selection, yielding highly active clones. Additional bands indicate the isolated clones were not specific towards the displayed peptide and that additional targets were also biotinylated (**Figure 4c**).

FIGURE AND TABLE LEGENDS:

Table 1: PCR reagents. Units and volumes may vary between manufacturers.

Table 2: Error-prone PCR reagents. Units and volumes may vary between manufacturers. * prepared in section 1 of the protocol.

Figure 1: The bacterial display system for BirA selection. (**a**) The system was based on the co-expression of 2 components: BirA and eCPX fused with the acceptor peptide (AP). eCPX is transported to the surface and, if the BirA variant biotinylates the AP, the biotin (red B) attached to the eCPX-AP is displayed on the surface. (**b**) The system was expressed from the plasmid pBAD-BirA-eCPX-AP, where BirA expression is controlled by an arabinose-inducible promoter and eCPX-AP expression is driven by the T7 promoter. (**c**) After generation of a randomly mutated library of BirA variants (step 1), BirA and eCPX-AP expression was induced (step 2). Bacteria were incubated with affinity reagent (step 3), unbound bacteria were discarded (step 4) and selected bacteria were amplified (step 5). This figure has been modified from Granhøj et al.[9]

Figure 2: Representative results from model selection with pBAD-BirA-eCPX-AP and pBAD-BirA-eCPX-AP(K10A). (**a**) By western blotting, eCPX-AP was observed to be biotinylated in both uninduced and induced bacteria, while no biotinylation of eCPX-AP(K10A) was detected even after the induction of BirA. BirA expression was detected by anti-6xHis antibody. * Indicates an unspecific streptavidin-reacting protein when BirA was induced. (**b**) Bacterial cultures with induced expression of BirA and eCPX-AP aggregate rapidly after addition of magnetic streptavidin-beads (arrow), while no aggregation was observed in AP(K10A) bacteria. (**c**) BirA was present in bacteria expressing eCPX-AP and eCPX-AP(K10A) before thestreptavidin-pulldown (input), but only BirA in eCPX-AP expressing bacteria was pulled down by streptavidin. In agreement, (**d**) viable bacteria were precipitated effective in eCPX-AP, but not eCPX-AP(K10A), expressing bacteria. This figure has been modified from Granhøj et al.[9]

Figure 3: Primer design and incorporation of target peptide coding sequence into pBAD-BirA-eCPX-AP by PCR. (**a**) An example of the primers design used for the incorporation of a peptide sequence derived from αENaC into the C-terminal of eCPX. The biotin accepting lysine is shown in red. The target peptide sequence was reverse translated to DNA, and forward and reverse primers were designed by ensuring a ~15 base overlap between the primers. (**b**) Representative agarose gel electrophoresis of PCR with pBAD-BirA-eCPX-AP as template and primers specific for α, β, and γ-ENaC derived peptide sequences, respectively. A clear and strong DNA product at ~5900 bp was indicative of a successful PCR. “M” indicates marker lane.

Figure 4: Representative results from selection and characterization of bacteria displaying peptides. Bacteria displaying a peptide derived from (**a**) TagRFP showed a clear enrichment

after 3 selection rounds, while a peptide derived from (**b**) EGFP showed no enrichment of streptavidin-bound bacteria even after 4 selection rounds. (**c**) 10 clones of bacteria displaying a peptide from γENaC through 5 selection rounds were tested for their ability to biotinylate the γENaC-peptide. All 10 clones showed a streptavidin-reacting band consistent with the size of eCPX-AP, indicating that the isolated clones contain BirA variants that biotinylate the displayed peptide. Additional streptavidin-reacting bands were also observed, indicating that other proteins, besides the displayed peptide, were also biotinylated. * Indicates an endogenous *E. coli* protein biotinylated by BirA. This figure has been modified from Granhøj et al.[9]

DISCUSSION:

As for all selection methods, the stringency of the washing steps is of utmost importance. Since bacteria do not need to be eluted from the beads before the amplification of the selected clones, the high affinity binding between biotin and streptavidin can be used instead of using lower affinity avidins, as previously done with the phage display system, for the selection of BirA variants[7,8]. This ensures that rare clones are selected and that non-biotinylated bacteria are discarded. Another advantage of using bacterial display, as compared to phage display, is that bacterial display is quantitative[11] and, therefore, allows for the selection of the bacteria based on the enzymatic activity.

In the protocol, we used MACS to select for bacteria creating a binary selection system based on the presence or absence of biotin on the surface. However, by using quantitative fluorescence activated cell sorting, instead, it should be possible to select for bacteria that express the most active variants of BirA. This will be important in the future development of the novel BirA variants as it will allow an effective selection for the most active BirA variants.

We have, so far, used the bacterial display of 14 different peptides and, of those, 13 produced a clear enrichment[9], indicating that our selection system provides a robust method to select for the novel BirA variants. In the current setup, we have only tested the selection of BirA variants that are active towards 15-amino acid peptides and, thereby we preferentially selected for the BirA variants that are active towards the primary sequence of the target protein. The targeted lysine can, however, be buried inside the 3D structure of a protein or not be otherwise accessible for BirA, yielding BirA variants that are not active against their target protein. A potential solution would be to display the larger protein fragment on eCPX. The eCPX scaffold is versatile with respect to the peptide display[11]; however, it is not known whether larger proteins can be displayed.

We used the selection system to isolate a BirA variant that biotinylates native TagRFP[9]. The tested BirA variant specifically biotinylated TagRFP on the targeted lysines, but the activity of the isolated variant was low[9]. Therefore, further rounds of directed evolution should be performed to improve its activity. The target peptide is in the C-terminus of TagRFP, where the structural similarity between the displayed peptide and the protein region is more likely. Bioinformatic analysis of all human and mouse proteins shows that ~75% of the proteins contain one or more lysine within their first and/or last 30 amino acids[9]. Thus, the bacterial display system of peptides can potentially be used to isolate active BirA variants towards a large

fraction of native proteins.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Polyak, S. W., Abell, A. D., Wilce, M. C. J., Zhang, L., Booker, G. W. Structure, function and selective inhibition of bacterial acetyl-CoA carboxylase. *Applied Microbiology and Biotechnology.* **93** (3), 983–992, doi:10.1007/s00253-011-3796-z (2011).
2. Schatz, P. J. Use of Peptide Libraries to Map the Substrate Specificity of a Peptide-Modifying Enzyme: A 13 Residue Consensus Peptide Specifies Biotinylation in Escherichia coli. *Nature Biotechnology.* **11** (10), 1138–1143, doi:10.1038/nbt1093-1138 (1993).
3. de Boer, E., Rodriguez, P. et al. Efficient biotinylation and single-step purification of tagged transcription factors in mammalian cells and transgenic mice. *Proceedings of the National Academy of Sciences.* **100** (13), 7480–7485, doi:10.1073/pnas.1332608100 (2003).
4. Tannous, B. A., Grimm, J., Perry, K. F., Chen, J. W., Weissleder, R., Breakefield, X. O. Metabolic biotinylation of cell surface receptors for in vivo imaging. *Nature Methods.* **3** (5), 391–396, doi:10.1038/nmeth875 (2006).
5. Howarth, M., Takao, K., Hayashi, Y., Ting, A. Y. Targeting quantum dots to surface proteins in living cells with biotin ligase. *Proceedings of the National Academy of Sciences.* **102** (21), 7583–7588, doi:10.1073/pnas.0503125102 (2005).
6. Michael Green, N. Avidin and streptavidin. *Avidin-Biotin Technology*. **184**, 51–67 (1990).
7. Fujita, S., Taki, T., Taira, K. Selection of an Active Enzyme by Phage Display on the Basis of the Enzyme's Catalytic Activity in vivo. *ChemBioChem.* **6** (2), 315–321, doi:10.1002/cbic.200400215 (2005).
8. Iwamoto, M., Taki, T., Fujita, S. Selection of a biotin protein ligase by phage display using a combination of in vitro selection and in vivo enzymatic activity. *Journal of Bioscience and Bioengineering.* **107** (3), 230–234, doi:10.1016/j.jbiosc.2008.11.009 (2009).
9. Granhøj, J., Dimke, H., Svenningsen, P. A bacterial display system for effective selection of protein-biotin ligase BirA variants with novel peptide specificity. *Scientific Reports.* **9** (1), 4118, doi:10.1038/s41598-019-40984-x (2019).
10. Rice, J. J. Bacterial display using circularly permuted outer membrane protein OmpX yields high affinity peptide ligands. *Protein Science.* **15** (4), 825–836, doi:10.1110/ps.051897806 (2006).
11. Rice, J. J., Daugherty, P. S. Directed evolution of a biterminal bacterial display scaffold enhances the display of diverse peptides. *Protein Engineering Design and Selection.* **21** (7), 435–442, doi:10.1093/protein/gzn020 (2008).

12. Stothard, P. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *BioTechniques.* **28**, 1102–1104 (2000). https://www.bioinformatics.org/sms2/rev_trans.html

Figure 1

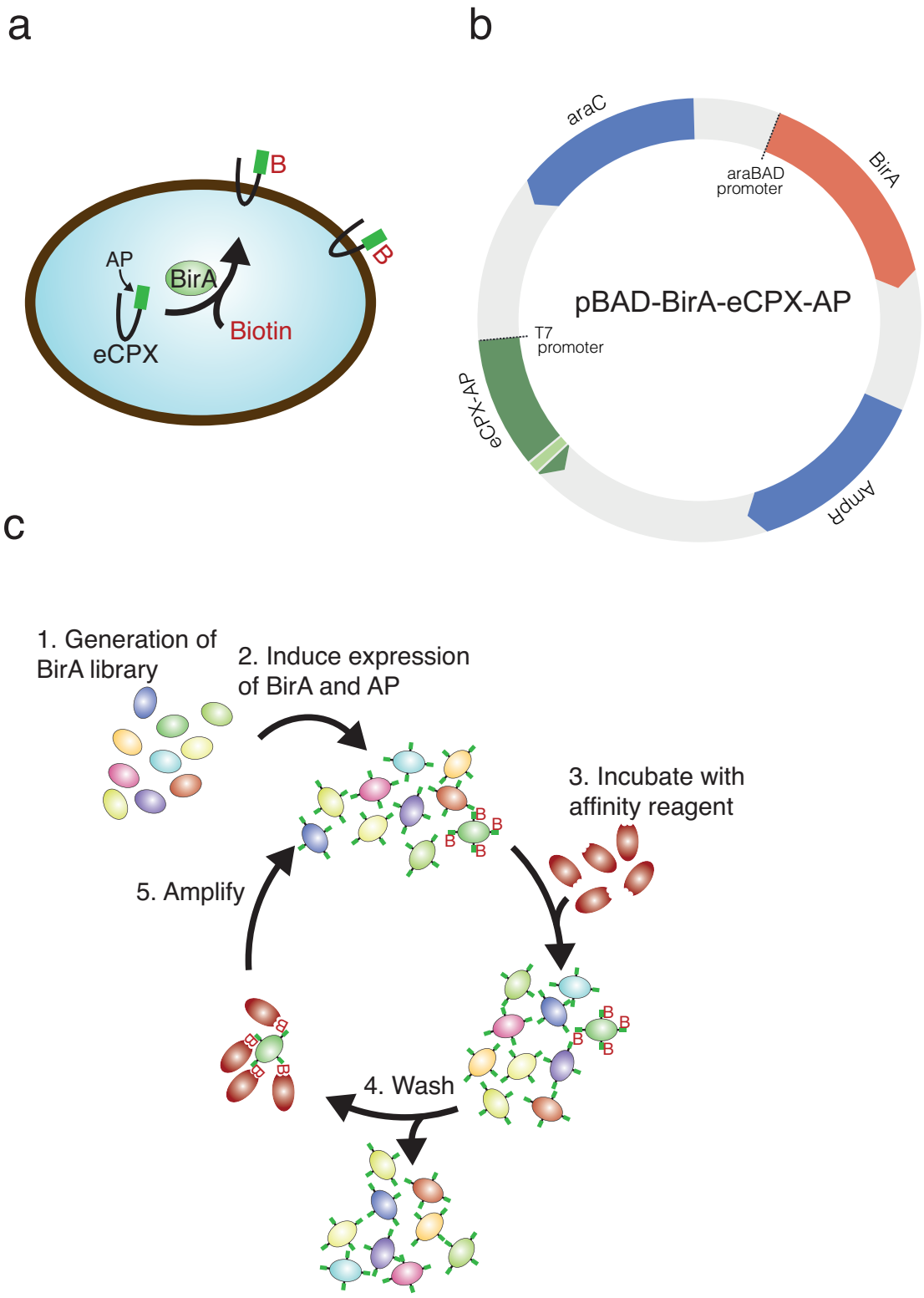
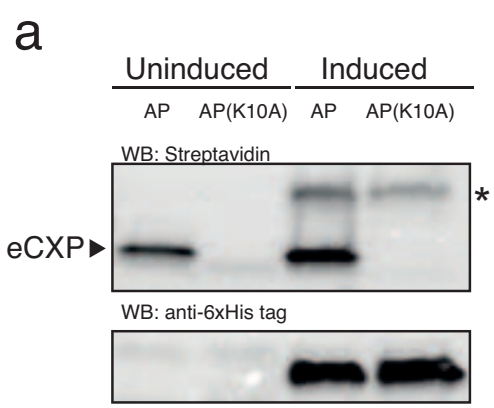
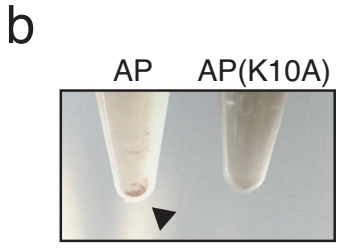
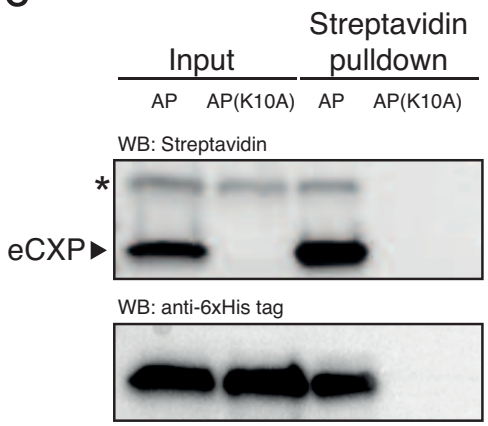


Figure 2







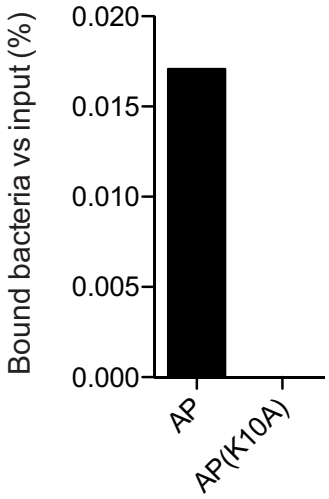


Figure 3

Figure 4

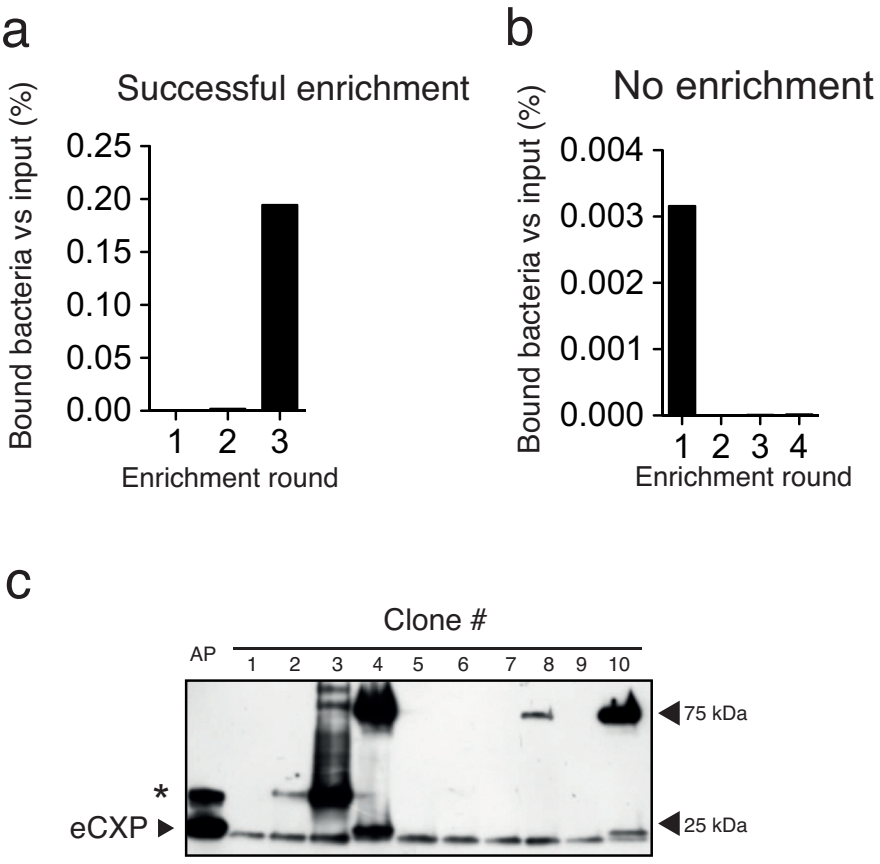


Table 1

Reagent	*volume (µL)*
5x Reaction Buffer	4
10 mM dNTP	0.4
10 µM Forward Primer	1
10 µM Reverse Primer	1
pBAD-BirA-eCPX-AP	Variable (~25 ng)
High-Fidelity DNA polymerase	0.20
Nuclease-Free Water	to 20

Table 2

Reagent	*volume (µL)*
2x Enzyme mix	25
pBAD-BirA-eCPX-AP with target peptide sequence*	Variable (~50 ng)
Mutant megaprimer	250 ng
Buffer	3
Nuclease-Free Water	to 50

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10% precast polyacrylamide gel	Bio-Rad	4561033	
Ampicilin	Sigma-Aldrich	A1593	
ApE - A plasmid editor v2.0	NA	NA	downloaded from http://jorgensen.bi
Arabinose	Sigma-Aldrich	A3256	
Biotin	Sigma-Aldrich	B4501	
DMSO	Sigma-Aldrich	D2650	
DPBS (10X), no calcium, no magnesium	ThermoFischer Scientific	14200083	
DpnI restriction enzyme	New England BioLabs	R0176	
Dynabeads MyOne Streptavidin C1	ThermoFischer Scientific	65001	
GenElute Plasmid Miniprep Kit	Sigma-Aldrich	PLN350	
GeneMorph II EZClone Domain Mutagensis kit	Agilent Technologies	200552	
Glucose	Sigma-Aldrich	G8270	
Glycerol	Sigma-Aldrich	G5516	
Immobilon-P PVDF Membrane	Millipore	IPVH15150	
IPTG	Sigma-Aldrich	I6758	
LS Columns	Miltenyi Biotec	130-042-401	
NaCl	Sigma-Aldrich	S7653	
NEB 5-alpha Competent E. coli	New England BioLabs	C2987	
NuPAGE LDS Sample Buffer (4X)	ThermoFischer Scientific	NP0007	
NuPAGE Sample Reducing Agent (10X)	ThermoFischer Scientific	NP0009	
pBAD-BirA-eCPX-AP	Addgene	121907	Used a template and positive control
pBAD-BirA-eCPX-AP(K10A)	Addgene	121908	negative control
Q5 High-Fidelity DNA Polymerase	New England BioLabs	M0491	For insertion of peptide sequence in p
QuadroMACS Separator	Miltenyi Biotec	130-090-976	
Skim Milk Powder	Sigma-Aldrich	70166	
Streptavidin-HRP	Agilent Technologies	P0397	

T7 Express lysY/Iq Competent E. coli	New England BioLabs	C3013
Tryptone	Millipore	T9410
Tween-20	Sigma-Aldrich	P9416
Western Lightning Plus-ECL	PerkinElmer	NEL103001EA
Yeast extract	Sigma-Aldrich	Y1625

iology.utah.edu/wayned/ape/

ɔBAD-BirA-eCPX-AP, any high fidelity polymerase will do

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Author(s):	Jeff Granhøj, Henrik Dimke, Per Svenningsen.

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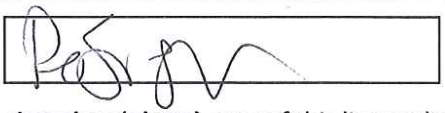
Signature:	
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Response:
We have carefully checked for spelling and grammar issues.

2. Please provide at least 6 keywords or phrases.
Response:
Done

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Response:
The embedded table has now been moved to Table 1.xlsx and is now accompanied by a title and description in the manuscript text.

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Response:
We have removed the commercial language as suggested.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
Response:
Done

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
Response:
Done

7. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
Response:
We have now moved the discussion of the step or added them as a “Note”.

8. Please ensure you answer the “how” question, i.e., how is the step performed?
Response:
We are not sure what is meant with this specific comment.

9. Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).
Response:
We have now added a description of how the software actions are performed.

10. Lines 92-95, 146-148, 186-188, 257-259, 284-287: Please convert into numbered action steps or make it a note. We cannot have paragraph of text in the protocol section.
Response:
Corrected

1.2: How is this done?
Response:
We have now added a description of how the software actions are performed.

4.3: LB/Ampicilin hvilken koncentration?

Response:
Changed to LB/Amp plates.

11. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response:

12. Please include a marker lane for PCR product gel.
Response:
Done.

13. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.
Response:
We have now inserted references in the Protocol section to the Figure that shows the experimental set-up, i.e. Figure 1c).

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Response:
Please find the link the editorial policy for use of image in the attached document in 4 paragraphs.

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response:
We have now explicitly covered the paragraphs

16. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. Please sort the materials table in alphabetical order.
Response:
Done

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript "Bacterial peptide display for selection of novel biotinylating enzymes" by Jeff Granhøj et al. is a protocol describing bacterial peptide display. Generally, it is written well in detail and steps are clear for readers. I suggest to accept it in the current form.
Response:
Thank you for your positive comment.

Reviewer #2:
Manuscript Summary:
In this methods paper, the authors provide detailed protocols for the discovery of novel mutants of biotin protein ligase (BirA) that can biotinylate non-native peptide sequences. There appears to be sufficient detail in the protocols as written for this style of publication. I have no major concerns and provide minor suggestions as ways to improve the clarity of the manuscript.

Major Concerns:
In Figure 2, the western blots have no molecular weight markers. Most journals expect to have the migration of markers shown on blots. I suspect that the non-specific band (*) may be BirA that has self biotinylated (Mw 35 kDa).
Response:
Thank you for your comments. We have now indicated the migration of MW markers on the western blot. The non-specific band could, indeed, be BirA (see response to the comment below for a statement addressing this concern).

Minor Concerns:
line 53, Should read "...development of new mutants of biotin protein ligases"
Response:
Thanks.

line 80, Should read "novel variants of E. coli BirA that .....
Response:
Thanks.

line 345, The endogenous protein is the biotin carboxyl carrier protein; a subunit of acetyl coA carboxylase.
Response:
Thanks. We inserted the following: (likely biotin carboxyl carrier protein; a subunit of acetyl coA carboxylase or self-biotinylated BirA)

line 353, Should read ".... streptavidin-reactive band .."
Response:
Thanks.

line 374, Should read "selection round. However, ..."
Response:
Thanks.

line 423. Include: The biotin accepting lysine is shown in red.
Response:
The sentence has now been inserted.

Figure 3 and b, the x-axis should contain a label (ie Enrichment round)
Response:
We have now labeled the x-axis' in panel a and b.

Reviewer #3:
Manuscript Summary:
This manuscript describes a protocol for generating new BirA enzymes that have an altered specificity motif and recognize a new particular linear peptide motif.
This is based on the Science Reports article published early in 2019 (Sci Rep. 2019; 9: 4118.)

Major Concerns:
Figures 1 and 2 are directly from another publication (Sci Rep. 2019; 9: 4118.) and should be remade, revised or not used at all.
Response:
Thank you for the comment. We have followed the author guidelines, which states that: "If a figure is adapted or republished from a previous publication, authors must cite the original article in the figure legend"
We have obtained permission to reuse the figures and inserted the statement: This figure has been modified from Granhøj et al.[9]

Minor Concerns:
268 Plate 100 µL of samples from "Input 10-6", "Input 10-8", "Input 10-10", "Output 10-2", "Output 10-3" and "Output 10-4" on LB/Ampicilin hvilken koncentration? plates
Response:
Thank you for making us aware of the mistake. We have now inserted: LB/Amp plates

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