Isolation of intact and active FoF1 ATP synthase using a FLAG-tagged subunit from the cyanobacterium *Synechocystis* sp. PCC 6803

The FoF1 ATP synthase (ATPase) is one of the most important protein complexes in energy metabolism. The isolation of functional ATPase complexes is fundamental to address questions about its assembly, regulation, and functions. This protocol describes the purification of intact and active ATPase from the model cyanobacterium *Synechocystis* sp. PCC 6803. Basis for purification is a 3xFLAG tag fused to the beta subunit. The ATPase is enzymatically active and its purity is demonstrated using mass spectrometry, denaturing, and blue-native PAGE.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

Isolation of intact and active FoF1 ATP synthase using a FLAG-tagged subunit from the cyanobacterium Synechocystis sp. PCC 6803

Kuo Song,1,4,6,* Stefan Tholen,2,3 Desirée Baumgartner,1,5 Oliver Schilling,2,3 and Wolfgang R. Hess1,7,*

1Genetics and Experimental Bioinformatics, Faculty of Biology, University of Freiburg, D-79104 Freiburg, Germany
2Institute for Surgical Pathology, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany
3Proteomic Core Facility (ProtCF), Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany
4Present address: Institute of Biochemistry and Molecular Biology, Faculty of Medicine, University of Freiburg, D-79104 Freiburg, Germany
5Present address: Hahn-Schickard, Georges-Koehler-Allee 103, 79110 Freiburg, Germany
6Technical contact
7Lead contact
*Correspondence: kuo.song@biochemie.uni-freiburg.de (K.S.), wolfgang.hess@biologie.uni-freiburg.de (W.R.H.)
https://doi.org/10.1016/j.xpro.2022.101623

SUMMARY

The FoF1 ATP synthase (ATPase) is one of the most important protein complexes in energy metabolism. The isolation of functional ATPase complexes is fundamental to address questions about its assembly, regulation, and functions. This protocol describes the purification of intact and active ATPase from the model cyanobacterium Synechocystis sp. PCC 6803. Basis for purification is a 3×FLAG tag fused to the beta subunit. The ATPase is enzymatically active and its purity is demonstrated using mass spectrometry, denaturing, and blue-native PAGE. For complete details on the use and execution of this protocol, please refer to Song et al. (2022).

BEFORE YOU BEGIN

This protocol guides the isolation of functional ATP synthase (ATPase) complexes from the cyanobacterium Synechocystis sp. PCC 6803 (Synechocystis 6803), based on the 3×FLAG tag (DYKDHD-G-DYKDHD-I-DYKDDDDK) fused to one of the exposed sites of the protein complex. ATP synthases produce ATP driven by proton or sodium gradients. The proton gradients can be generated by respiration or photosynthesis (Kühlbrandt, 2019).

Cyanobacteria are unique in combining photosynthetic and respiratory electron transport chains in the same membrane system, the thylakoids (Mullineaux, 2014), where also the ATPase complexes are located. While we developed and tested the protocol in the Synechocystis 6803 model strain (Song et al., 2022), we anticipate that the underlying principle should work in a wide range of other organisms. In addition to the purification of ATPase, this protocol can also be applied to the isolation of FLAG-tagged proteins for other purposes with slight modifications, such as co-immunoprecipitation as described also in Song et al. (2022).

Note: We used kanamycin and chloramphenicol resistances for selection of engineered cyanobacteria and promoters that can be controlled by the available concentration of Cu2+ ions, but different combinations of selection markers and promoters can be utilized.
**Synechocystis 6803 mutant construction**

**© Timing: 2–4 weeks**

1. The 3×FLAG tag should be fused to an exposed site of the ATPase complex, for example in this case, we used a strain containing a 3×FLAG tag fused to the C terminus of the ATPase subunit beta.

   **Note:** Since the genes encoding ATPase subunits are mostly organized in operons, the direct insertion of the 3×FLAG tag coding sequence might interfere with the expression or regulation of the respective ATPase subunit gene and its neighboring genes. Therefore, here, we overexpressed the 3×FLAG-tagged ATPase subunit beta from a second gene copy located on conjugative vector pVZ322 (Zinchenko et al., 1999), while keeping the original genomic gene copy uninterrupted. We show that this strategy is productive for the isolation of the ATPase.

   **Note:** For Synechocystis 6803, this overexpression strategy using a replicating plasmid vector also saves time compared to the integration of gene sequences into the chromosome by homologous recombination requiring repeated selection cycles until full segregation is achieved.

2. We mentioned the construction of this plasmid in our publication Song et al. (2022). To tag and bring atpB, the gene encoding ATPase subunit beta, under control of the Cu²⁺-inducible promoter of the petE gene (Bovy et al., 1992; Zhang et al., 1992), the sequence upstream of petE was fused to the atpB reading frame without the stop codon, followed by the 3×FLAG coding tag, a TAG stop codon, the atpT 3′ UTR and the bacteriophage lambda oop transcription terminator (see Figure 1 for details). The following three fragments were amplified by PCR using the indicated specific primers:
   a. The petE promoter fragment using the primer pair P1/P2;
   b. The atpB coding sequence with P3/P4;
   c. The 3′ segment (3×FLAG tag, TAG, atpT 3′ UTR, Toop) with the primers P5/P6 and using the plasmid pEX-A2-PpetE::norf1OE-3×FLAG as template, which was generated by gene synthesis (Baumgartner et al., 2016).

3. We combined the generated fragments via Gibson Assembly (Gibson et al., 2009) using plasmid pUC19 as a vector backbone. The finished construct was released by restriction digests with XbaI and PstI, re-ligated into vector pVZ322 (Zinchenko et al., 1999) and transferred into Synechocystis 6803 via triparental mating with E. coli strains J53/RP4 and TOP10F’ (Scholz et al., 2013). Transconjugants were selected on BG11 agar containing 10 μg mL⁻¹ gentamicin and 50 μg mL⁻¹ kanamycin.

   **Alternatives:** Instead of using vector pVZ322, a plasmid of the recently developed pSOMA shuttle vector series (Opel et al., 2022) might be used.
Cell cultivation and collection

- **Timing**: 1–2 weeks for steps 4 and 5
- **Timing**: 1 h for steps 6 and 7

4. *Synechocystis* 6803 should be cultured under optimal growth conditions in BG11 medium (Rippka et al., 1979) to ensure an abundance of ATPase complexes exists in vivo.
   a. For cultivation, 500 mL Erlenmeyer flasks could be used, filled with up to 300 mL of BG11 medium and kept under an illumination of 50 photons m$^{-2}$ s$^{-2}$ white light with a continuous stream of air bubbled through the medium.
   b. If an inducible promoter is used for the overexpression of FLAG-tagged ATPase subunit, the expression could be induced at early to middle exponential phase. We have used the Cu$^{2+}$-responsive PpetE promoter and therefore cultivated the cells initially in copper-free BG11. To induce expression of the 3×FLAG-atpB gene from the PpetE promoter, CuSO$_4$ was added to a final concentration of 2 μM.
   c. For *Synechocystis* 6803, at least 5 OD of cells are suggested for one purification (e.g., collecting 5 L of cultures at an OD750 of 1.0).
   d. Alternatively to the cultivation in flasks bubbled with air, the Cell-DEG system (Lippi et al., 2018) was found suitable for culturing *Synechocystis* 6803 rapidly to high cell densities and for subsequent ATPase purification.

5. Cells should be collected before or shortly after they enter stationary phase (by centrifugation at 5,000 × g for 10 min), and then washed at least once using FLAG buffer (Jakob et al., 2020). The collected cell pellets should be used immediately or can be kept at -80°C until use.

**Alternatives:** We have kept the collected cell pellets up to one week at −80°C without seeing detrimental effects on ATPase activity, but recommend to keep storage time as short as possible.

6. Make sure to prepare sufficient amounts of membrane extraction buffer A and FLAG buffer: about 20 mL of buffer A and 120 mL FLAG buffer are needed for each purification.
7. Prepare anti-FLAG beads according to the manufacturer’s instructions. For this, about 15 mL of 0.1 M glycine-HCl (pH 3.5) will be needed.

**Note:** Anti-FLAG M2 magnetic beads and Anti-FLAG M2 agarose beads (both from Sigma-Aldrich) were tested in this protocol, both have worked similarly well.

---

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** |
| Polyclonal anti-AtpB antibody 1:2000 dilution | Agrisera | Cat#AS05 085-10 |
| Mouse anti-FLAG M2 antibody 1:5000 dilution | Sigma-Aldrich | Cat#A8592-1MG |
| Goat anti-rabbit secondary antibody 1:2000 dilution | Sigma-Aldrich | Cat#A8275 |
| **Bacterial and virus strains** |
| Synechocystis sp. PCC 6803 (PpetE-atpB-3×FLAG) | (Song et al., 2022) | N/A |
| **Chemicals, peptides, and recombinant proteins** |
| 3×DYKDDDDK Peptide | Pierce™ | CAT#A36805 |
| Anti-FLAG™ M2 Affinity Gel | Sigma-Aldrich | CAT#A2220 |
| Anti-FLAG™ M2 Magnetic Beads | Sigma-Aldrich | CAT#M8823 |
| cOmplete™ Protease Inhibitor Cocktail Tablets | Sigma-Aldrich | CAT#04693132001 |
| n-Dodecyl-β-D-maltoside (DDM) | PanReac AppliChem | CAT#A08190005 |

(Continued on next page)
Continued

### MATERIALS AND EQUIPMENT

#### FLAG buffer

| Reagent                           | Final concentration | Amount   |
|----------------------------------|---------------------|----------|
| Hepes-NaOH adjusted to pH 7.0     | 50 mM               | 2.38 g   |
| MgCl₂ (2 M)                      | 5 mM                | 0.5 mL   |
| CaCl₂ (1 M)                      | 25 mM               | 5 mL     |
| NaCl (5 M)                       | 150 mM              | 6 mL     |
| Glycerol                         | 10%                 | 20 mL    |
| Total                            | N/A                 | Add to 200 mL with ddH₂O |

Store at −20°C

Complete Protease Inhibitor Add before use, 1 tablet per 50 mL buffer.

#### Membrane extraction buffer A

| Reagent                           | Final concentration | Amount   |
|----------------------------------|---------------------|----------|
| Hepes-NaOH adjusted to pH 7.0     | 20 mM               | 0.238 g  |
| Betaine                          | 1.0 M               | 5.85 g   |
| MgCl₂ (2 M)                      | 15 mM               | 0.375 mL |
| CaCl₂ (1 M)                      | 15 mM               | 0.075 mL |
| D-sorbitol                       | 0.4 M               | 3.64 g   |
| 6-amino-n-caproic acid (2 M)     | 1 mM                | 0.025 mL |
| Total                            | N/A                 | 50 mL    |

Store at 4°C for up to 1 month

Complete Protease Inhibitor Add before use, 1 tablet per 50 mL buffer.
**STEP-BY-STEP METHOD DETAILS**

**Membrane preparation and solubilization—Day 1**

**Timing:** 5–7 h

ATP synthases are membrane complexes. In *Synechocystis* 6803, the ATPase locates mainly to the thylakoid membrane. Therefore, preparation and solubilization of the membrane fraction is an important initiation step for the purification of ATPase.

1. Resuspend the collected *Synechocystis* 6803 cells with buffer A (Store at 4°C for up to 1 month; take 10 mL buffer A for 5 OD of cells), break cells using Precellys 24 homogenizer with 6 cycles of 25 s at 6,000 rpm in the presence of glass beads.

   **Note:** We use a bead volume of approximately 1/3 to 1/2 of the resuspension volume.

   **Note:** The following steps should be kept at 4°C or on ice, unless specifically mentioned.

   **Alternatives:** Cells can be lysed via multiple methods, including lysis by mechanical vibration as in the Precellys 24 or Retsch homogenizer (Retsch GmbH), as well as high pressure cell disruptors.

   **Alternatives:** cOmplete™ Protease Inhibitor used in this protocol can be replaced by other protease inhibitors, such as phenylmethylsulfonyl fluoride (PMSF).

2. Centrifugation at 4,000 × g for 10 min, then remove the pellet fraction including unbroken cells, cell debris, and beads.
3. Centrifugation at 20,000 × g for 1 h, remove the supernatant, and keep the pellet fraction which is the crude membranes.

   **Optional:** To increase the purity of the collected membranes, the collected membranes can be washed for 1–2 times using buffer A, by first re-suspend the pellet from step 3, then repeat step 3. This can further remove soluble proteins and avoid unspecific binding as much as possible.

   **Pause point:** The membrane pellet can be stored at −80°C (better less than 1 month). Avoid repeated freeze-thaw cycles.

4. Re-suspend the collected membrane pellet thoroughly using FLAG buffer (Store at −20°C), and incubate on ice for 0.5–1 h to solubilize the membranes.

   **Note:** Brief gentle vortexing may help but we didn’t test this.

5. Measure the protein concentration of the membrane solution, and adjust the protein concentration to 5–10 mg/mL with FLAG buffer.

   **Note:** For measuring the protein concentration, here the Coomassie Plus Bradford assay was used.

   **Alternatives:** Other assays such as the Pierce BCA protein assay may be suitable as well for protein quantification.
Note: Adjustment of the protein concentration is important for sufficient solubilization of the membranes.

6. Slowly add dodecylmaltoside (DDM) to a final concentration of 1% (w/v) from freshly prepared 10% DDM solution in water, incubate for 1 h with gentle agitation to solubilize the membrane.

7. Centrifuge the membrane solution at 20,000 g for 30 min to remove insoluble debris, and filter the resulting supernatant through 0.45 μm membrane.

Optional: Depending on the actual volume, the filtered solution can be concentrated at this step, to give a proper volume for further flag tag affinity chromatography. Higher protein concentration can lead to better binding efficiency, and save time if several loading rounds are desired to obtain higher product amounts. We used 100 KDa cutoff Amicon Ultra-2, Merck Millipore ultra-centrifugal filters to concentrate the solution from 5 OD of cells to 10 mL volume.

Pause point: The solubilized fraction can be stored at 4°C for up to 12 h, or at −80°C for longer periods (better less than 1 month). The protein complexes are more fragile without intact membrane, therefore freeze-thaw cycles are not recommended here.

FLAG-tag based affinity chromatography—Day 2

© Timing: 2–4 h

The affinity chromatography based on flag tag and anti-FLAG beads can be used to isolate FLAG-tagged proteins/protein complexes at small scale (for co-immunoprecipitation, followed by mass spectrometry analysis) or larger scale (for biochemistry analysis). Here the anti-FLAG agarose beads were used; the anti-FLAG magnetic beads can also be used, with similar purity. The protocol described here can be applied for co-immunoprecipitation with slight modifications, which will be described in notes.

8. Preparation of Anti-FLAG M2 affinity resin (Sigma-Aldrich CAT#A2220; for this step, one can also refer to the manufacturer’s instructions).
   a. Re-suspend the affinity resin thoroughly, and transfer appropriate amount of resin into a clean, empty chromatography column (for 5 OD of Synechocystis 6803 cells, 1 mL of resin is recommended).
   b. Drain the gel bed naturally, followed by washing using three sequential column volumes (CV) of 0.1 M glycine-HCl (pH 3.5) (do not leave the resin in glycine-HCl for longer than 20 min).
   c. Wash the resin with 5 CV of FLAG buffer to equilibrate the resin, and the resin is then ready for use.

△ CRITICAL: No extra pressures other than the gravity are recommended during preparation of affinity resins, as well as during following chromatography steps; this can promote the formation of channels, ensure sufficient contact between solutions and resins, and avoid bubbles. Also, like other resins, the gel bed should always be kept wet.

9. Load the membrane lysate onto the prepared column under gravity flow; the flow through membrane lysate sample are reloaded twice to increase binding.

Note: Repeated loading is especially important for co-immunoprecipitation assay.

Optional: Repeat the loading-washing-elution procedure if you have limited bed volume but want to accumulate more proteins.
10. Sufficiently wash the column with 5–15 times of 2 CV of FLAG buffer 2 [FLAG buffer supplemented with 0.03% (w/v) DDM; prepare before use].

△ CRITICAL: The washing step 10 is very important for the purity of the final product. Here, 0.03% (w/v) DDM was added to the FLAG buffer, in order to remove lipids from the purified ATPase, and to increase the solubility of intact ATPase complex. Also, the buffer volume used for washing should be sufficient; in the case of FLAG-tagged ATPase, ten times of 2 CV of washing buffer, which is the highest volume recommended in the manufacture’s instruction, still resulted in quite high amounts of unspecific proteins as indicated by SDS-PAGE; these unspecific proteins became mostly undetectable when 15 times of 2 CV washing buffer was used.

Note: The additional supplementation of 0.03% (w/v) DDM is not necessary for the purification of non-membrane embedded proteins, or for co-immunoprecipitation assays in which no membrane-located protein interaction partners are expected.

11. Elute the FLAG-tagged ATPase with 2 times of 1.5 CV of FLAG buffer 2 supplemented with 150 μg/mL 3×FLAG peptide.

Alternatives: The resulting eluate can be further concentrated using 100 KDa cutoff ultra-centrifugal filter; this can not only remove most of the 3×FLAG peptide and overexpressed ATPase subunit, but also allow the changing of buffer.

Alternatives: The elution step 11 can be performed at a temperature of about 22°C–25°C, with eluted samples kept on ice.

Note: Instead of FLAG buffer 2 containing 3×FLAG peptide, 0.1 M glycine HCl (pH 3.5) can be used for the elution of FLAG-tagged proteins, but its effect on the activity of ATPase was not tested. For co-immunoprecipitation assays, both 0.1 M glycine HCl (pH 3.5) or SDS-PAGE sample buffer can be considered for elution instead of the 3×FLAG peptide used here; in that case the eluted samples can proceed directly to SDS-PAGE, followed by mass spectrometry to identify protein components.

Purity check and following biochemical assays

© Timing: variable

Before conducting any biochemistry analysis, the integrity and purity of the isolated ATPase should be checked. Generally, SDS-PAGE, Western blot, and mass spectrometry are recommended sequentially to control the quality of isolated ATPase.

12. SDS-PAGE analysis of the isolated ATPase.

Note: The molecular weight judged from SDS-PAGE is inaccurate and should only be used as reference.

13. Western blot analysis of the isolated ATPase.

Optional: The isolated ATPase can also be checked using native PAGE such as blue-native PAGE (Wittig et al., 2006). Only one band of about 400–600 kDa should be visible.

14. Checking the isolated ATPase using mass spectrometry.
Note: By this method one can be sure about the purity of the isolated ATPase, as well as the possible presence of contaminations that might interfere following biochemical assays.

15. Conduct biochemical assays as needed, such as the ATP hydrolysis assay described in Song et al. (2022).

**Liquid chromatography-tandem mass spectrometry**

© Timing: 3–5 days

Samples were prepared using S-Trap micro filters (Protifi, Huntington, NY) following the manufacturer’s procedure. Afterwards peptides were analyzed on a Q-Exactive Plus mass spectrometer (Thermo Scientific, San Jose, CA) coupled to an EASY-nLCTM 1000 UHPLC system (Thermo Scientific). Other LC-MS/MS systems might be adequate as well but cannot be addressed here.

Sample preparation:

16. Samples were mixed 1:1 with 2× SDS protein solubilization buffer (10% SDS, 100 mM triethylammonium bicarbonate, pH 7.55; store at −20°C) for a final concentration of 5% SDS.

Note: At least 2% final concentration of SDS is necessary in order for the S-Trap to bind the proteins.

17. Samples were reduced by adding 5 mM TCEP (Tris(2-carboxyethyl) phosphine hydrochloride) and incubated for 10 min at 95°C.

18. Samples were alkylated by adding iodacetamide to a final concentration of 10 mM and incubated for 20 min at a temperature of about 22°C–25°C in the dark.

19. 12% aqueous phosphoric acid were added at 1:10 for a final concentration of ~1.2% phosphoric acid (e.g., 2.5 mL into 25 μL) and mixed.

20. At least 6 times the current sample volume of S-Trap protein binding buffer (90% aqueous methanol containing a final concentration of 100 mM triethylammonium bicarbonate, pH 7.1; store at −20°C) were added.

Note: Given sufficient protein amount, the solution will appear translucent.

21. The S-Trap micro column was placed in a 2 mL tube to collect the flow through. Afterwards, the acidified sample was pipetted onto the micro column.

Note: If initial sample volume is higher than one column load, load the column multiple times until the full volume has passed the column material. Up to 100 μg protein can be loaded.

22. The micro column was centrifuged at 4,000 g until all sample has passed through the S-Trap column.

Note: Proteins will be trapped within the protein-trapping matrix of the spin column. After each centrifugation step, make sure that all added solution has gone through the S-Trap column. Do not exceed 5,000 g with the S-Trap mini spin columns.

23. Captured protein has been washed by adding 150 μL S-Trap protein binding buffer; centrifugation was repeated. The whole washing procedure was repeated three times.

24. The S-Trap micro column with trapped sample was moved to a clean 1.5 mL low-bind sample tube for the digestion.
25. 20 μL of Digestion buffer (50 mM triethylammonium bicarbonate) containing trypsin at 1:10–1:25 (wt:wt) were added onto the top of the micro column.

*Note:* For effective digestions, do not apply less than 0.75 μg of trypsin. The protein trap is hydrophilic and will absorb the solution. Ensure there is no air bubble between the protease digestion solution and the protein trap.

26. The S-Trap column was capped to limit evaporative loss.

27. Incubation for 1 h at 47°C.

28. Peptide elution was carried out in three subsequent elution steps all into the same tube:
   a. 40 μL of 50 mM Digestion buffer were added and centrifuged at 4,000 x g for approx. 1 min to elute peptides.
   b. 40 μL of 0.2% aqueous formic acid were added and centrifuged at 4,000 x g for approx. 1 min to elute peptides a second time.
   c. 35 μL of 50% acetonitrile containing 0.2% formic acid were added and centrifuged at 4,000 g for approx. 1 min to elute peptides a third/last time.

29. The peptide solution was completely dried in a SpeedVac. Dried peptides were resuspended in 25 μL H2O and a BCA assay performed to determine the peptide concentration.

**MS measurement and data analysis:**

30. One ug of peptides was analyzed on a Q-Exactive Plus mass spectrometer (Thermo Scientific, San Jose, CA) coupled to an EASY-nLCTM 1000 UHPLC system (Thermo Scientific). The analytical column was self-packed with silica beads coated with C18 (Reprosil Pur C18-AQ, d = 3 Å) (Dr. Maisch HPLC GmbH, Ammerbusch, Germany).

31. For peptide separation, a linear gradient of increasing buffer B (0.1% formic acid in 80% acetonitrile, Fluka) was applied, ranging from 5 to 40% buffer B over the first 90 min and from 40 to 100% buffer B in the subsequent 30 min (120 min separating gradient length).

32. Peptides were analyzed in data dependent acquisition mode (DDA). Survey scans were performed at 70,000 resolution, an AGC target of 3e6 and a maximum injection time of 50 ms followed by targeting the top 10 precursor ions for fragmentation scans at 17,500 resolution with 1.6 m/z isolation windows, an NCE of 30 and a dynamic exclusion time of 35 s. For all MS2 scans the intensity threshold was set to 1.3e5, the AGC to 1e4 and the maximum injection time to 80 ms.

33. Raw data were analyzed with MaxQuant (v 1.6.14.0) allowing two missed cleavage sites, no variable modifications, carbamidomethylation of cysteines as fixed modification. The database “Synechocystis sp. (strain PCC 6803 / Kazusa)” was downloaded from https://www.uniprot.org/proteomes/UP000001425 on Jan 29th, 2021. Only unique peptides were used for quantification using the iBAQ (Intensity Based Absolute Quantification) algorithm.

*Note:* iBAQ values calculated by MaxQuant are the (raw) intensities divided by the number of theoretical tryptic peptides. Thus, iBAQ values are proportional to the molar quantities of the proteins. The iBAQ algorithm can roughly estimate the relative abundance of the proteins within each sample (Nagaraj et al., 2011; Schwanhäuser et al., 2011).

**EXPECTED OUTCOMES**

About 50 μg ATPase can be obtained per OD of cells following this protocol. Higher yields can be obtained by increasing the amounts of anti-FLAG beads or the number of binding-elution cycles.

Fractions obtained during the ATPase purification following this protocol were analyzed using SDS-PAGE (Figure 2A). Although some ATPase was washed off from the column before elution (lane 3), and some was retained on the column (lane 5), the vast majority of the ATPase was successfully
purified (lane 4). Lane 6 proved the 100 kDa cutoff ultra-centrifugal filter to be efficient to get rid of most of the over-expressed AtpB-3×FLAG and the 3×FLAG peptide used for elution, as discussed in the alternative in step 11.

Additionally, the isolated ATPase can also be checked using blue-native PAGE (Wittig et al., 2006). Only one band between 480 kDa and 720 kDa was observed in our protocol (Figure 2B), proving that the isolated ATPase was an intact protein complex.

The protein components of the isolated ATPase (lane 4 in Figure 2A) can be precisely identified by mass spectrometry (Table 1). The ATPase components showed the highest iBAQ scores, which underlines the enrichment and high purity of the isolated ATPase.

The membrane located ATPase subunit C was not identified. A possible explanation could be the short peptide sequence of this subunit. From a short protein sequence, only a small number of tryptic peptides can be expected and these may not have ideal characteristics for MS measurement. Therefore, absence of this subunit from the MS results does not necessarily mean it was not present in the sample, it rather was not detected during the MS measurement. If necessary, this problem can be overcome by using instead of trypsin other proteases in the digestion in step 25. This will result in peptides of different sequences and different characteristics for MS measurement.

**LIMITATIONS**

The purification efficiency of the 3×FLAG tag is relatively lower compared with the 6×His tag, meaning that more anti-FLAG M2 agarose beads (or anti-FLAG M2 magnetic beads) or more purification cycles will be needed if more final product is required. To purify protein complexes such as ATPase using 3×FLAG tag, the tag sequence should be fused to an appropriate exposed location. Once solubilized from the membrane, the ATPase is stable at ambient temperature (about 22°C–25°C) only for less than 2 h, and freeze-thaw cycles significantly destroy its activity; the buffers used in this protocol may be optimized to achieve higher stability.

**TROUBLESHOOTING**

**Problem 1**

No FLAG tagged ATPase subunit was expressed (step 4).
Table 1. Top 15 most abundant protein components in the purified ATPase detected by mass spectrometry

| Fasta headers | Mol. weight [kDa] | iBAQ     |
|---------------|------------------|----------|
| sp|P27179|ATPA_SYNY3 ATP synthase subunit alpha OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=atpA PE=3 SV=1 | 53.965 | 35201000000 |
| sp|P27181|ATPF_SYNY3 ATP synthase subunit b OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=atpF PE=3 SV=2 | 19.804 | 30421000000 |
| sp|P26527|ATPB_SYNY3 ATP synthase subunit beta OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=atpB PE=3 SV=1 | 51.732 | 28091000000 |
| sp|P17253|ATPG_SYNY3 ATP synthase gamma chain OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=atpG PE=3 SV=1 | 34.605 | 16847000000 |
| sp|P26533|ATPE_SYNY3 ATP synthase epsilon chain OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=atpE PE=1 SV=3 | 14.58 | 14808000000 |
| sp|P27180|ATPD_SYNY3 ATP synthase subunit delta OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=atpD PE=3 SV=1 | 20.093 | 13668000000 |
| sp|P27178|ATP6_SYNY3 ATP synthase subunit a OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=atpB PE=3 SV=1 | 30.698 | 28800000000 |
| sp|P27183|ATPF2_SYNY3 ATP synthase subunit b OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=atpF PE=3 SV=1 | 16.245 | 15663000000 |
| sp|P73790|PHS_SYNY3 Putative pterin-4-alpha-carbinolamine dehydratase OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=BAA17842 PE=3 SV=1 | 10.734 | 6386400000 |
| trj|P74433|PSAL_SYNY3 Photosystem I reaction center subunit XI OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=psaL PE=1 SV=2 | 34.864 | 1243200000 |
| sp|Q54714|PHCA_SYNY3 C-phycocyanin alpha subunit OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=pcpA PE=1 SV=1 | 18.126 | 4501500000 |
| sp|P37277|PSAL_SYNY3 Photosystem I reaction center subunit XI OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=psaL PE=1 SV=1 | 16.624 | 4282500000 |
| sp|Q54715|PSAF_SYNY3 Photosystem I reaction center subunit III OS=Synechocystis sp. (strain PCC 6803 / Kazusa) OX=1111708 GN=psaF PE=1 SV=1 | 18.249 | 2110400000 |

Potential solution

- Check the plasmid and strain if they were correctly constructed, or if there were mutations that interfere with its expression;
- Check if the FLAG-tagged protein was correctly induced; in this case, the growth of Synechocystis cells in copper-free medium is important for the subsequent induction of the P_{pdxE} promoter;
- If FLAG tag is not commonly used in your strain platform, consider to use another tag.

Problem 2

The FLAG tagged ATPase subunit was expressed, but no ATPase was purified at the end in steps 12 or 13.

Potential solution

- Estimate the expression level of your recombinant protein, it is possible that the purified ATPase is not detectable if the expression level is too low;
• Check the efficiency of your beads, and make sure sufficient amounts of beads were used for purification;  
• Check in which fraction the FLAG tagged protein occurs: whether it is in the flow-through fraction (check the beads) or was washed out during the wash step (check the buffer conditions).

**Problem 3**
The yields in purified ATPase were much lower than indicated in the “expected outcomes” section of this protocol.

**Potential solution**
• If you have used another strain other than Synechocystis 6803, or expressed a FLAG-tagged ATPase subunit using another promoter, different levels of FLAG-tagged ATPase might have led to the lower yield. In this case, try alternative promoters or vectors.  
• If the same system was used: make sure that the FLAG-tagged subunit was properly induced, that the cells were collected before or shortly after entering stationary phase and verify that most FLAG-tagged ATPase was bound to the column by checking the flow through and wash fractions.

**Problem 4**
Too much contaminations in the purified ATPase (steps 12–14).

**Potential solution**
• Check the efficiency of the anti-FLAG magnetic or agarose beads, use new beads if necessary;  
• Make sure to re-suspend all the beads with washing buffer during each wash of step 10;  
• Include a mock purification using control strain containing no FLAG-tagged protein, in order to estimate the degree of background contaminants.

**Problem 5**
No ATP hydrolysis activity was detected using the purified ATPase (step 15).

**Potential solution**
As mentioned in the limitation part, the stability of the isolated ATPase is relatively low, and the buffer system may need further improvement. Therefore, it is recommended that the activity assay is conducted immediately after purification; if not possible, freeze and thaw the purified ATPase only once by splitting the prepared ATPase sample into suitable aliquots.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wolfgang R. Hess (wolfgang.hess@biologie.uni-freiburg.de).

**Materials availability**
All unique/stable reagents generated in this study are available from the lead contact with a completed materials transfer agreement.

**Data and code availability**
The accession number for the mass spectrometry proteomics datasets reported in this study is at the MassIVE repository: [http://massive.ucsd.edu/](http://massive.ucsd.edu/); dataset identifier: MSV000089593. Related mass spectrometry raw data ([Song et al., 2022](http://proteomecentral.proteomexchange.org/PXD034273)) have been deposited at the ProteomeXchange Consortium: [http://proteomecentral.proteomexchange.org/PXD034273](http://proteomecentral.proteomexchange.org/PXD034273). This study did not generate new code for analysis.
ACKNOWLEDGMENTS

This study was funded by the German Research Foundation (DFG) priority program SPP2002 “Small Proteins in Prokaryotes, an Unexplored World” (grant HE 2544/12-2 to W.R.H.) and the research training group BioInMe 322977937/GRK2344 to K.S., O.S., and W.R.H., and by a China Scholarship Council grant to K.S. The Proteomic Platform – Core Facility was supported by the Medical Faculty of the University of Freiburg to O.S. (2021/A3-Sch).

AUTHOR CONTRIBUTIONS

D.B. constructed the strain with the atpB-3×FLAG construct. K.S. carried out the molecular-genetic and biochemical analyses in Synechocystis 6803. S.T. and O.S. performed proteomics analyses. W.R.H. designed the study, and all authors analyzed the data. K.S. and W.R.H. drafted the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Baumgartner, D., Kopf, M., Klähn, S., Steglich, C., and Hess, W.R. (2016). Small proteins in cyanobacteria provide a paradigm for the functional analysis of the bacterial micro-proteome. BMC Microbiol. 16, 285. https://doi.org/10.1186/s12868-016-0896-z.

Boy, A., de Vrieze, G., Borrias, M., and Weisbeek, P. (1992). Transcriptional regulation of the plastocyanin and cytochrome c553 genes from the cyanobacterium Anabaena sp. PCC 7118. Mol. Microbiol. 6, 1507–1513.

Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., and Smith, H.O. (2011). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6, 343–345. https://doi.org/10.1038/nmeth.1318.

Jakob, A., Nakamura, H., Kobayashi, A., Sugimoto, Y., Wilde, A., and Masuda, S. (2020). The (PATAAN)-CheY-like response regulator PixE interacts with the motor ATPase PilB1 to control negative phototaxis in the cyanobacterium Synechocystis sp. PCC 6803. Plant Cell Physiol. 61, 296–307. https://doi.org/10.1093/pcp/pcz194.

Kuhlbrandt, W. (2019). Structure and mechanisms of F-type ATP synthases. Annu. Rev. Biochem. 88, 515–549. https://doi.org/10.1146/annurev-biochem-013118-110903.

Lippi, L., Bähr, L., Wüstenberg, A., Wilde, A., and Steuer, R. (2018). Exploring the potential of high-density cultivation of cyanobacteria for the production of cyanophycin. Algal Res. 37, 363–366. https://doi.org/10.1016/j.algal.2018.02.028.

Mullineaux, C.W. (2014). Co-existence of photosynthetic and respiratory activities in cyanobacterial thylakoid membranes. Biochim. Biophys. Acta 1837, 503–511. https://doi.org/10.1016/j.bbadis.2013.11.017.

Nagaraj, N., Wisniewski, J.R., Geiger, T., Cox, J., Kircher, M., Kelso, J., Paabo, S., and Mann, M. (2011). Deep proteome and transcriptome mapping of a human cancer cell line. Mol. Syst. Biol. 7, 548. https://doi.org/10.1038/msb.2011.81.

Opel, F., Siebert, N.A., Klatt, S., Tüllinghoff, A., Hantke, J.G., Toepel, J., Bühler, B., Nürnberg, D.J., and Klähn, S. (2022). Generation of synthetic shuttle vectors enabling modular genetic engineering of cyanobacteria. ACS Synth. Biol. 11, 1758–1771. https://doi.org/10.1021/acssynbio.1c00605.

Rippka, R., Stanier, R.Y., Deruelles, J., Herdman, M., and Waterbury, J.B. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. Microbiology 111, 1–61. https://doi.org/10.1099/00221287-111-1-1.

Schwanhäusser, B., Busse, D., Li, N., Dittrig, G., Schuchhardt, J., Wolf, J., Chen, W., and Selbach, M. (2011). Global quantification of mammalian gene expression control. Nature 473, 337–342. https://doi.org/10.1038/nature10098.

Song, K., Baumgartner, D., Hagemann, M., Muro-Pastor, A.M., Maaß, S., Becher, D., and Hess, W.R. (2022). Atpθ is an inhibitor of FoF1 ATP synthase to arrest ATP hydrolysis during low-energy conditions in cyanobacteria. Curr. Biol. 32, 136–148.e5. https://doi.org/10.1016/j.cub.2021.10.051.

Wittig, I., Braun, H.-P., and Schägger, H. (2006). Blue native PAGE. Nat. Protoc. 1, 418–428. https://doi.org/10.1038/nprot.2006.62.

Zinchenko, V.V., Piven, I.V., Melnik, V.A., and Shestakov, S.V. (1999). Vectors for the complementation analysis of cyanobacterial mutants. Russ. J. Genet. 35, 228–232.