

S1. Detailed methods and materials for development of protein extractions, global antibodies and protein standards.

We describe here the methods used to develop and validate quantitative immunodetection systems and reagents for Photosystems I and II, RuBisCO, ATP synthase, Nitrogenase and Glutamine synthetase. We implemented the immunoquantitation approach because other methods require more sample, more complicated specific assay conditions and because it complements alternate functional approaches by providing good proxies for maximum capacity. It is indeed possible to measure the approximate activities of a variety of enzymes and catalytic complexes directly. Each assay, however, requires a separate sub-sample, usually of fresh material, whose size and handling requirements of which can constrain or complicate an experiment, especially one involving field samples.

Immunoquantitations can be performed on flash-frozen samples, and are not susceptible to the bottle or incubation time effects associated with specific activity assays (eg. for carbon or nitrogen fixation). Measuring photosynthetic complexes by fluorescence or oxygen evolution, moreover, requires specialized equipment and is susceptible to sample handling and incubation effects.

Quantitative immunodetection requires three components: protocols for consistent extraction of proteins from samples; antibodies for detection of the target protein, and standards for calibrating the detection. Implicit in the application of immunoquantitation is the assumption that equal detection of members of a protein family can be achieved using antibodies elicited toward peptides having amino acid sequences that are conserved across the protein family. A perfect match between the peptide immunogen used to create

antibodies and the corresponding amino acid sequence in the target protein ensures a consistent signal on immunoblots, even if the target proteins are extracted from multiple species. A perfect match between the target sequence in a protein standard and that in the protein extracted from samples then allows accurate quantitation of the target protein through comparison of the antibody binding signal for the target protein and the cognate protein standard.

The immunoquantitation approach allows us to use a single small, frozen sample extracted into a single denaturing extraction buffer for the parallel detection of multiple protein subunits. This is a considerable advantage in comparison to performing individual assays under special, native conditions for each of the separate complexes, which would require more sample and multiple sample preparation protocols.

Extraction of samples

Several methods for disruption of cells were compared, including sonication (Model 450, Branson Ultrasonics), repeated freeze/thaws in the presence of detergent, and micro-beadbeating using either a vortex-mounted system or a Fastprep (MoBio) machine. Total protein and chlorophyll concentrations were used as indicators of cell disruption, while the intensity of representative membrane (PsbA) and soluble (RbcL) protein signal on immunoblots, detected as described in Materials and methods, showed the extent of extraction of these proteins. Sonication combined with freeze/thaw cycles proved to be the most effective extraction method.

Sonications were initially performed in 3ml volumes in borosilicate tubes submerged in a stirred ice bath. The excessive sonication times required to achieve maximum

extraction in these large volumes caused the progressive degradation of both RbcL (Fig. S.1) and PsbA, and possibly cross-linking of RbcL.

To minimize sonication times and to lower the sample volumes to experimentally practical levels, sample cell pellets in 2 mL conical-bottom polypropylene tubes were resuspended in 150 to 500 μL denaturing extraction buffer (EDTA 0.5 mmol L^{-1} , Tris base 140 mmol L^{-1} , Tris HCl 105 mmol L^{-1} , Glycerol 10%, Pefabloc 0.1mg/ml, LDS 2%). This volume decrease was a notable improvement. We have refined our method such that we can perform approximately ten separate immunoquantitations using a single sample containing as little as 30 μg of total protein (approximately 0.3 μg to 2 μg chla), assuming a 2 μg protein load per minigel lane.

Sample cell pellets and the appropriate volume of denaturing extraction buffer were frozen in liquid nitrogen and thawed by sonication with a micro-tip attachment at a power setting of 30%. To avoid excessive sample heating, sonication was stopped and samples refrozen immediately once fully thawed. Extraction efficiencies were estimated by analyzing equal volumes of extracts from successive rounds of sonication on immunoblots. Measuring PsbA and RbcL band intensities as a function of sonication cycle, we estimated that extraction efficiencies for PsbA and RbcL were between 90% and 95%. The combination of liquid N_2 freeze/thaws and shorter duration, higher intensity sonication resulted in improved yields of both PsbA and RbcL and less degradation, particularly of RbcL, compared to longer duration sonications in larger volumes. *Synechocystis* PCC 6803, for instance, was disrupted to over 90% through sonication/freeze thaw cycling in 150 to 500 μL of denaturing extraction buffer but required extensive sonication in 3 mL volumes to achieve even 75% disruption.

The number of sonication-freeze thaw cycles was optimized for each taxon. For *Synechococcus elongatus* Sp. PCC 7942, for example, three rounds was optimal, generating maximum extraction of PsbA and RbcL. Many taxa were nearly entirely disrupted by one (*Heterosigma*, *Thalassiosira*, *Trichodesmium*) or two (*Euglena*, *Chlamydomonas*) rounds of sonication and freezing.

Selection of peptide sequences for generation of antibodies

Antibodies were designed for detection of key photosynthetic complexes across wide taxonomic ranges. Photosystems I and II, RuBisCO, ATP synthase, Cytochrome b6f and bacterial Glutamine synthetase and Nitrogenase are multisubunit protein complexes. One or more representative subunits were thus chosen (Table S.1) as immunodetection targets for each complex. Amino acid sequences of the chosen subunits were obtained from the National Center for Biotechnology Information (NCBI) public database (<http://www.ncbi.nlm.nih.gov/>) for organisms from each taxonomic group for which we wished to detect the subunit. We generally included representative protein sequences from cyanobacteria, red algae, green algae, diatoms, raphidophytes, dinoflagellates and higher plants. Protein sequences were aligned using Multalin (<http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html>), allowing the identification of regions perfectly conserved across the subunit sequences of the representative taxa. Amino acid sequences seven to fifteen residues in length were chosen as candidate peptide tags. These tags were subjected to a BLAST (basic local alignment and search tool) search, which compared submitted peptide sequences to all known protein sequences, thus allowing the

conservation of the submitted sequence to be checked and the taxonomic range of conservation to be defined. BLAST searches also revealed unrelated but potentially cross-reacting proteins containing similar epitopes.

Production and testing of antibodies

Agrisera Ab in Sweden performed antibody production. Peptides were synthesized according to the defined amino acid sequence tags. Peptides were coupled through a terminal cysteine to a carrier protein, keyhole limpet hemocyanin, and injected into either chickens or rabbits. Blood sera or egg yolks were collected at several time-points and tested for the presence of antibodies specific to the target protein. The first test of antibody specificity was the detection of proteins of the predicted size in protein extracts from representative photoautotrophs. Total protein extracts from a panel of photosynthetic organisms were separated by electrophoresis and transferred to PVDF membranes, as described in Methods and materials. Fig. S.2 shows representative immunoblots for RbcL, NifH, AtpB, PsaC and PsbA subunits detected from total protein samples extracted from *Trichodesmium* sp. Purified native (RbcL) and recombinant (all other subunits) were included as positive controls for the molecular weight and specificity of the immunodetections. For immunoquantitation of the subunits such immunoblots were performed on protein extracts from 4-5 independent replicate *Trichodesmium* samples.

Positive detections were followed by competition assays, also known as neutralization assays. In these tests, antibody preparations were first diluted (1:5000 to

1:50000) in TBST. One half of the diluted antibody solution was pre-incubated with a large (approximately 100X) molar excess of the free peptide antigen toward which antibodies were initially raised. Antigen binding sites in this solution were thus occupied by peptide and rendered unavailable for binding to target proteins on blots. The other half of the antibody solution was not exposed to peptide antigen. To prepare blots, duplicate samples of total protein from several representative photoautotrophs were run on two halves of a polyacrylamide gel and transferred to a PVDF membrane. The halves of the membrane were sliced apart after blocking, but prior to incubating with the two antibody solutions, plus and minus peptide, in parallel. The detection of a band on the membrane incubated with antibody solution and the absence of that band on the membrane incubated with peptide-neutralized antibody solution confirmed that the band contained the epitope of interest. A representative example of a competition assay showing the specificity of the PsaC immunodetection is shown in Fig. S.3. Similar assays were performed for RbcL, NifH, AtpB, and PsbA.

Generation of Protein Standards

RbcL standard was produced from partially purified RuBisCO (Sigma). Recombinant protein standards for PsaC, PsbA, PsbB, AtpB, NifH and GlnA were produced using the *in vitro* transcription/translation RTS500 kit from Roche Diagnostics GmbH.

To produce recombinant protein subunits, genomic DNA from exponentially growing cyanobacterial cultures was obtained using the UltraClean Microbial DNA Isolation Kit (MoBio 12224-50). The DNA from 3.6 ml of cell suspension was eluted in 50 μ L of

ddH₂O. 0.5µL of genomic DNA solution was used for polymerase chain reaction (PCR) amplification of the coding regions of *psaC*, *psbA2*, *psbB*, *atpB*, and *glnA* genes from *Synechocystis* Sp. PCC 6803 and *nifH* from *Nostoc* Sp. PCC 7120 (Table S.1). PCR product sizes were analyzed by agarose gel electrophoresis. The following primer pairs were used (engineered restriction sites are underscored):

psaC primers for N-terminal His6 tag (Topoisomerase ligated; no restriction sites added),
fwd - CGCTTAATTAACATATGACCTCCCATAGTGTA AAAAATTTACG and
rev - TTAGTTAGTTACCGGATCCCTTAGTAAGCTAAACCCATACTGC

psbA primers for C-terminal His6 tag,
fwd - NdeI ATTATTCATATGACAACGACTCTCCAACAGCG
rev - SacI ACATAGGAGCTCGACCGTTGACAGCAGGAGCGG

psbB primers for C-terminal His6 tag,
fwd - NcoI ATTATTCATGGGACTACCTTGGTATCGCG
rev - SacI ACATAGGAGCTCGGGCTTCTTTCCGGGTGGA

atpB primers for C-terminal His6 tag,
fwd - PciI GACGATACATGTTAGCCGTA AAAAGAAGCAACTAACGTTG
rev - SacI ACATAGGAGCTCGACCCTCTTTGAGCTTGGCACC

glnA primers for C-terminal His6 tag
fwd - NdeI ATTATTATTCATATGGCCAGAACCCCCCAGGAAG
rev - SacI ACATAGGAGCTCGGCAGTCGTAGTACAAGGAGAATTTCG

nifH primers for C-terminal His6 tag
fwd - NcoI ATTATTCATGGCTGACGAAAACATTAGACAGATAG
rev - SacI ACATAGGAGCTCGTTTGGTAGCTTCTGCGGGCTTAC

PsaC was cloned into TOPO vector pCR 2.4 (Invitrogen) using topoisomerase mediated ligation of the PCR product into the vector. When expressed from this vector, PsaC was fused to an N-terminal sequence of six histidine residues (His6 tag). For all other standards, restriction sites were designed into primers so that products could be cloned into pIVEX 2.3d or 2.4d expression vectors (Roche Diagnostics), which contain regulatory sequences for transcription and translation and create fusion proteins with a C-terminal or N-terminal His6 tags. Plasmids containing cloned sequences were sequenced (Genome Quebec Sequencing Platform) to confirm that target sequences and regulatory regions were correct.

Purification of Standards

Recombinant proteins were purified from the translation mixture using Ni-NTA agarose resin affinity chromatography. The pellet fraction containing the recombinant protein was solubilized in 8 mol L⁻¹ urea in 0.5 mol L⁻¹ NaCl, 20 mmol L⁻¹ sodium phosphate pH 7.8. Ni-NTA agarose resin (Invitrogen) was washed twice in ddH₂O. Resin was added to the solubilized protein, and the mixture was mixed gently on a rotary mixer for 30 minutes. The resin was settled by centrifugation at 700 x g for 1 minute. The supernatant was removed by gentle aspiration. The resin was washed with 2 wash buffers: 8 mol L⁻¹ urea, 0.5 mol L⁻¹ NaCl, 20 mmol L⁻¹ sodium phosphate pH 6.0, then pH 5.3. Elution of the protein was performed in 8 mol L⁻¹ urea, 0.5 mol L⁻¹ NaCl, 20 mmol L⁻¹ sodium phosphate pH 4.0. The resin was then stripped with 1X LDS denaturing extraction buffer

(EDTA 0.5 mmol L⁻¹, Tris base 140 mmol L⁻¹, Tris HCl 105 mmol L⁻¹, Glycerol 10%, Pefabloc 0.1mg/ml, LDS 2%). The recombinant protein was tracked in the fractions by immunoblotting with mouse anti-His6 horseradish peroxidase conjugated antibody (Cedar Lanes) and, when available, with antibodies specific for the peptide sequence tag in the protein subunit. The bulk of the protein was obtained in the denaturing extraction buffer fraction. These immunoblots also showed the extent, if any, of protein truncation, which may occur during protein synthesis.

Purity of protein subunits in fractions was checked using SDS-PAGE followed by staining of the gel with SafeStain (Invitrogen). The cleanest fractions were pooled. Several different volumes were then loaded onto SDS-PAGE gels adjacent to lanes containing known quantities of bovine serum albumin (BSA) and RbcL as quantitation standards. Gels were stained with SafeStain and fully destained. The amount of material in all bands was measured by densitometry with using ImageQuant software (BioRad). Standard curves of BSA and RbcL were used to determine recombinant subunit quantities.

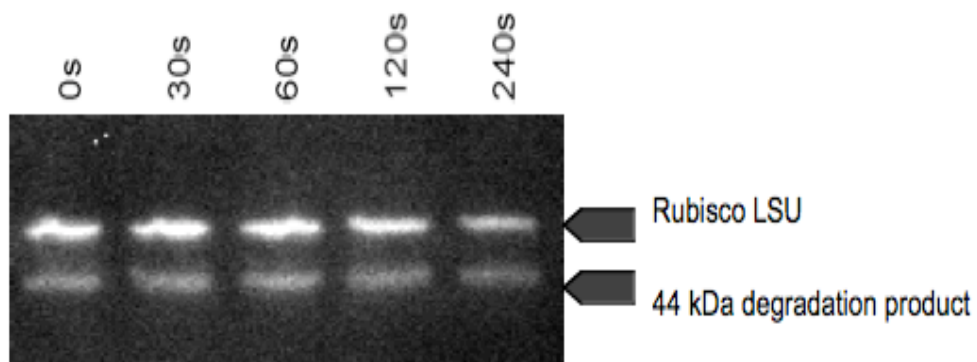


Fig. S.1 Loss of RbcL protein by multiple cycles of sonication.

Purified RuBisCO protein was suspended in denaturing extraction buffer and then sonicated for multiple cycles of 30s with intervening freezing in liquid N₂. RbcL was detection by immunoblotting with an anti-RbcL antibody (Agrisera).

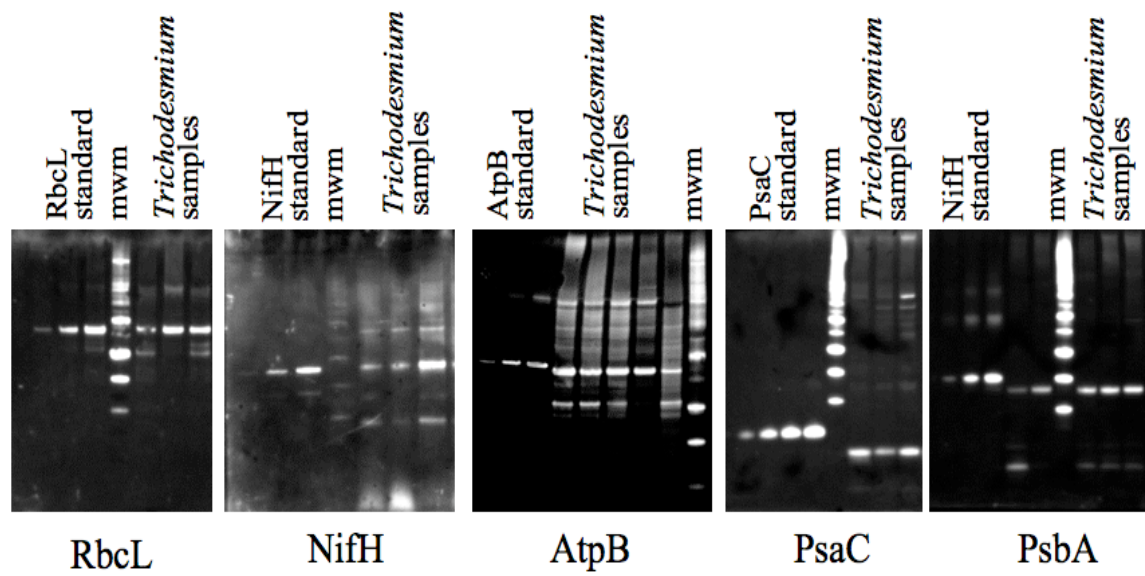


Fig. S.2 Immunoblots with global antibodies detecting RbcL, NifH, AtpB, PsaC and PsbA proteins from total protein extracted from *Trichodesmium*. Recombinant standards are slightly larger than the natural proteins due to the presence of a 2.6 kDa terminal His6 tag. mwm: molecular weight marker lane

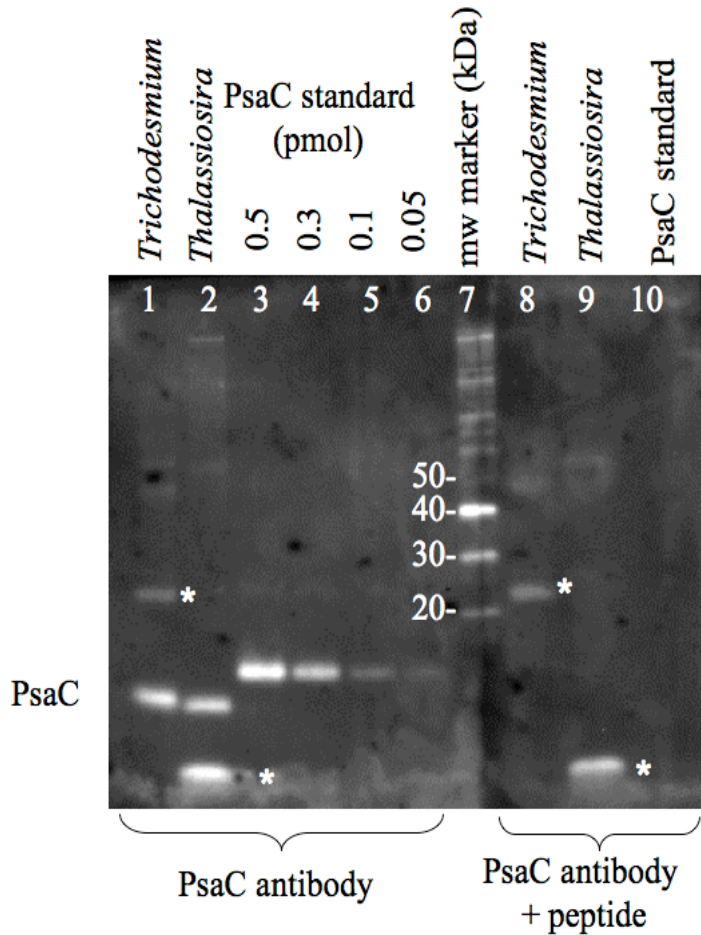


Fig. S.3 Immunoblot of PsaC in the diatom *Thalassiosira* and in the cyanobacterium *Trichodesmium* (lanes 1,2), showing PsaC protein of approximately 8.8 kDa. Recombinant PsaC protein, used as quantitation standard and positive control (lanes 3,4,5,6), is 11.4 kDa, 2.6 kDa larger than the native protein due to the presence of an N-terminal His6 tag. In the competition assay (lanes 8,9,10), PsaC antibodies were preincubated with the peptide to which antibodies were raised, The PsaC protein is not detected in these lanes, indicating the specificity of the antibody to the target peptide sequence in the PsaC protein. Asterisks indicate nonspecific detections or cross-reactions. mwm: molecular weight marker lane