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AOX | AOX positive control/quantitation standard

Qty: AS04 054S

AS04 054S | Protein standard for quantitation and positive control

This product is sold out.

Price:

Agrisera Western Blot protocol and video tutorials

Protocols to work with plant and algal protein extracts

Quantitative Immunoblotting in Plant Sciences using Global Antibodies

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Summary:
Detecting and quantitating important proteins in plants and other photosynthetic organisms has become more practical and powerful with the application of quantitative immunoblotting using global antibodies which recognize proteins evenly across a wide range of taxa. While many tools are commercially available for the examination of medically relevant proteins in mammals, such tools have been lacking for the study of pressing questions in other organisms. Here we present the advantages of quantitative immunoblotting and the methodology necessary to apply these tools.

Introduction | The exquisite specificity of the antibody antigen interaction has long been exploited by protein biochemists. Antibodies bind to small regions on proteins, termed epitopes, which often consist of only a few amino acids. This binding is highly specific
and of high affinity. As each antibody recognizes only a small part of the whole protein, an immunogenic peptide can be selected using sequence alignments. This short peptide is highly conserved and exclusive to the protein under study. The resulting antibodies are then immunoreactive with the conserved peptide region within intact proteins derived from a number of species. We refer to these as Global Antibodies, and they provide both scientific and practical advantages. In non-medical areas such as plant sciences, comparative analyses across species, is often productive; **Global Antibodies** permit such cross-taxon studies with level detection efficiencies across wide taxonomic ranges. From a practical standpoint, a Global Antibody functioning across a wide taxonomic range reduces the cost of antibody production and stocking by creating a detection tool useful in a wide range of studies.
The ELISA, or Enzyme Linked ImmunoSorbent Assay, employs specific antibodies in a high through put application for the measurement of specific substances in samples of diverse origins. While some sample and antibody combinations can be optimized for detections using ELISA, others are less successful. This can be the result of antibodies with significant cross reactivities, samples that do not adhere optimally to the plate, a lack of quantitated standards or samples of variable quality that display differential reactivity in ELISA. A similar approach is the quantitative immunoblot. As such, it uses the same principles as the ELISA, a reliance on the highly specific antibody antigen interaction to measure the amount of a given protein in a complex mixture. The
major distinguishing feature of this method is the inclusion of a step to electrophoretically separate the proteins in the sample according to their molecular weights. Thus, all samples can be solubilized using a standardized procedure and analyzed concurrently for a number of interesting proteins. The separation step allows the researcher to be more confident that the signal generated actually results from the target protein in question because it is spatially resolved from cross-reactions which would confound an ELISA system.

Limitations of Traditional Techniques | A number of traditional techniques are available for the measurement of different functions or complexes of photosynthetic organisms. These include an array of enzyme and functional assays. A

disadvantage of such approaches is that they require individually specialized sample harvesting and preparation techniques, and often specialized equipment or reagents for each assay. Therefore a single sample preparation cannot be used to quantify different protein complexes. Furthermore, many samples are not available in the quantities or preservation states necessary for multiple determinations of different protein complexes.

Advantages of Immunodetections with Global Antibodies | When Global Antibodies are used for quantitative immunodetections even small, mixed samples from various harvesting procedures can be assessed for multiple complexes simultaneously. Since all detections employ a common solubilization protocol as a starting point, comparisons between complexes can be made with greater confidence. Since Global Antibodies recognize short highly conserved regions of the protein targets they can be applied to a very broad range of target organisms. They can also be applied with considerable confidence to uncharacterized or mixed samples.
Quantitated recombinant protein standards have been generated to be used as a calibration with Global Antibodies. The target peptide antigen is perfectly conserved in these standard proteins, thus they can be applied for comparative quantitation of the target protein from all species.

Standards and antibodies available

Photosystem I Anti-PsaC (AS04 042) | standard: recombinant PsaC(AS04 042S)
Photosystem II Anti-PsbA C-terminal (AS01 016, AS05 084) | Anti-PsbA N-terminal | (AS06 124) | standard: recombinant PsbA (AS01 016S) | Anti-PsbB (AS04 038) | Anti-PsbD (AS06 146) |
ATP Synthase Anti-AtpB (AS03 030, AS05 085) | standard: recombinant AtpB (AS03 030S) |
RuBisCO Anti-RbcL RbcL (AS01 017, AS03 037)| standard: Rubisco protein (AS03 017S)
Nitrogen Metabolism: Anti-GlnA (AS01 018) | standard: recombinant GlnA (AS01 018S) |
Anti-NifH (AS01 021A) | standard: recombinant NifH (AS01 021S)

Methodology | Sample Preparation. Plant samples are generally ground with liquid nitrogen in a mortar and pestle. The resulting powder is transferred to a plastic tube. Algal samples can be either concentrated by centrifugation or, preferably, by filtration onto glass fiber filters.
Solubilization is performed in Agrisera extraction buffer PEB (AS08 300) , 0.1mg/mL PefaBloc SC (AEBSF) protease inhibitor (Roche). Disruption is most optimally obtained through flash freezing of the sample in liquid nitrogen alternated with thawing by sonication with a microtip. This process can be repeated depending on the toughness of the sample. The sample is adjusted to 50 mM dithiothreitol and heated to 70°C for 5 minutes. Samples are cooled and centrifuged briefly prior to electrophoresis. Optimal quantitation is achieved using moderate sample loads per gel lane, generally 0.5 to 2.5 ug total protein, depending on the abundance of the target protein.
Electrophoresis and Immunoblotting: Once solubilized, the proteins can be separated electrophoretically in a number of systems. We obtain optimal results with the Invitrogen NuPAGE gel system using Bis-Tris 4-12% gradient gels. Proteins are separated in MES SDS running buffer according to the manufacturer's recommendations at 200 V for 35 minutes. The gels are transferred to PVDF in the same apparatus, the SureLock XCell blot module, for 60 minutes at 30 V for a single gel or 80 minutes for a pair. Following transfer the blots are blocked in 2% blocking agent in tris buffered saline with 0.1% Tween 20 (TBS-T) for 1 hour at room temperature with gentle agitation. The blot is incubated with primary antibody, usually at 1:25 000 to 1:50 000 diluted in 2% blocking agent, for 1 hour at room temperature. For quantitation a relatively high primary antibody:target protein ratio gives more reliable results than immunoblots at low ratios of primary antibody:target protein.
The blot is washed extensively in TBS-T (twice briefly, once for 15 minutes and three times for five minutes). The blot is incubated with secondary antibody, for example goat anti-rabbit IgG horse radish peroxidase conjugated, at 1:50,000 in 2% blocking agent, for 1h/RT with agitation. The blot is washed as above and developed with chemiluminescence detection reagent in mid picogram or extreme femtogram range.

Quantitation | When quantitated standards are included on the blot, the samples can be quantitated using the available software. We obtain excellent quantitation with images captured on the Bio-Rad Fluor-S-Max or equivalent instrument using Bio-Rad QuantityOne software. The contour tool is used to select the area for quantitation and the values are background subtracted to give an adjusted volume in counts for each standard and sample. Using this protocol allows us to generate linear standard curves over 1-1.5 orders of magnitude range in target load. It is important to note that immunodetections usually show a strongly sigmoidal signal to load response curve, with a region of trace detection of low loads, a pseudolinear range and a region of saturated response with high loads. For immunoquantitation it is critical that the target proteins in the samples and the standard curve fall within the pseudolinear range. Our total detection range using this protocol spans over 2 orders of magnitude, but the quantifiable range is narrower.

Example | Undergraduate students performed a light shift experiment on two cyanobacterial strains, *Synechococcus elongatus* PCC 7942 and *Anabaena* sp. PCC 7120. The students prepared 4 samples for each species and loaded 0.1 ug chlorophyll per lane. Standard lanes of PsbA (D1) protein contained 0.05 pmoles , 0.15 pmoles and 0.45 pmoles. The blots were processed as described above with the chicken anti-PsbA antibody used at 1:20,000 and rabbit anti-chicken IgY-HRP secondary antibodies at 1:50,000. The resulting blot is shown in Figure 1.

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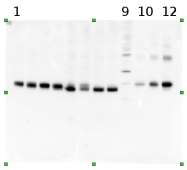


Figure 1: An example immunoblot of samples of *Synechococcus elongatus* PCC 7942 (lanes 1-4) and *Anabaena* sp. PCC 7120 (lanes 5-8). Molecular weight markers (MagicMark XP, Invitrogen) are in lane 9. Recombinant PsbA protein standards are loaded in lanes 10-12 at 0.05 pmoles, 0.15 pmoles and 0.45 pmoles.

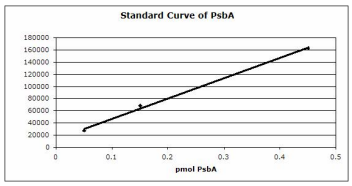


Figure 2: Standard curve for the blot in Figure 1. Picomoles of quantitated standard is plotted on the X-axis versus the adjusted signal volume obtained on the Y-axis.

Conclusion | Quantitative immunoblotting is a powerful but simple tool for the measurement of protein complexes from photosynthetic and other organisms. This technique can be applied to a wide range of species with excellent results, because Global Antibodies raised against conserved peptides can be shared across research on different species and research areas like: ecophysiology, microbial ecology, molecular physiology, proteomics and oceanography.

References | Bouchard J, Suzanne Roy S, Campbell DA (2006) Ultraviolet-B Effects on the Photosystem II-D1 Protein of Phytoplankton Species and Natural Phytoplankton Communities Photochemistry and Photobiology, 82: 936-951
Campbell DA, Cockshutt AM, Porankiewicz-Asplund J (2003) Analyzing Photosynthetic Complexes in Uncharacterized Species or Mixed Phytoplankton Communities using Global Antibodies. Physiologia Plantarum 119: 322-327
MacKenzie TDB, Johnson JM, Cockshutt AM, Burns RA, Campbell DA (2005) Large reallocations of carbon, nitrogen and photosynthetic reductant among phycobilisomes, photosystems and Rubisco during light acclimation in Synechococcus elongatus are constrained in cells under low environmental inorganic carbon. Archives of Microbiology, 183: 190 - 202

Recommended secondary antibodies: goat anti-rabbit HRP conjugated, goat anti-rabbit ALP conjugated

Recommended chemiluminescent detection reagent: AgriseraECLBright

Agrisera Western Blot protocol and video tutorials

Questions? You are welcome to contact us.

- Product Info

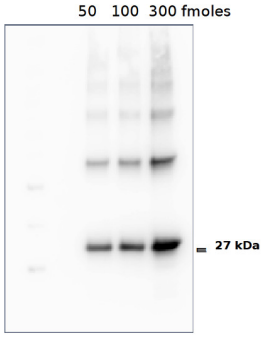
• Format:	Lyophilized
Quantity:	120 µl
Reconstitution:	For reconstitution add 100 µl of milliQ water. Please note that this product contains 10% glycerol and might appear as liquid but is provided lyophilized.
Storage:	Store lyophilized/reconstituted at -20°C; once reconstituted make aliquots to avoid repeated freeze-thaw cycles. Please, remember to spin tubes briefly prior to opening them to avoid any losses that might occur from lyophilized material adhering to the cap or sides of the tubes.
Tested applications:	Western blot (WB)
Recommended dilution:	Standard curve: 3 loads are recommended (0.5, 2 and 4µl). For most applications a sample load of 0.2 µg of chlorophyll/well will give a RbcL signal in this range.

Positive control: a 2 µl load per well is optimal for most chemiluminescent detection systems. Higher standard concentration needs to be used to allow detection by Coomasie stains. Such gels with higher standard concentration can not be used for quantitation using chemiluminescence.

This standard **is stabilized and ready** and does not require heating before loading on the gel. Please note that this product contains 10% glycerol and might appear as liquid but is provided lyophilized. Allow the product several minutes to solubilize after adding water. Mix thoroughly but gently Take extra care to mix thoroughly before each use, as the proteins tend to settle with the more dense layer after freezing.

Expected | apparent MW: 27 kDa

- Application Examples
- **Application example**



Following standard western blot procedure this image was obtained using a CCD imager (FluorSMax, Bio-Rad) and Quantity One software (Bio-Rad) as described below. Note: Optimal quantitation is achieved using moderate sample loads per gel lane, generally 0.5 to 2.5 ug total protein, depending on the abundance of the target protein.

Quantitation: When quantitated standards are included on the blot, the samples can be quantitated using the available software. Excellent quantitation can be obtained with images captured on the Bio-Rad Fluor-S-Max or equivalent instrument using Bio-Rad QuantityOne software. The contour tool is applied to the area for quantitation and the values are background subtracted to give an adjusted volume in counts for each standard and sample. Using this protocol linear standard curves are generated over 1-1.5 orders of magnitude range in target load. It is important to note that immunodetections usually show a strongly sigmoidal signal to load response curve, with a region of trace detection of low loads, a pseudolinear range and a region of saturated response with high loads. For immunoquantitation it is critical that the target proteins in the samples and the standard curve fall within the pseudolinear range. Our total detection range using this protocol spans over 2 orders of magnitude, but the quantifiable range is narrower.

Quantitative western blot: detailed method description.

- Additional Information
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Additional information: The AOX calibrated protein standard can be used in combination with Agrisera global anti-AOX antibodies (AS04 054) to quantitate AOX from a wide range of species. Global antibodies are raised against highly conserved amino acid sequence.

Quantitative western blot: detailed method description, video tutorial

Concentration: 0.1 pmol/µl. After re-constitution with sterile milliQ water, the final concentration of the AOX monomer is.0.1 pmol/µl. While a dimer is present in the lane, only the 27 kDa monomer contributes to the calibration.

Additional information (application): **Protein standard buffer composition:** Glycerol 10%, Tris Base 141 mM, Tris HCl 106 mM, LDS 2%, EDTA 0.51 mM, SERVA® Blue G250 0.22 mM, Phenol Red 0.175 mM, pH 8.5, 0.1mg/ml PefaBloc protease inhibitor (Roche), 50 mM DTT.

This standard is ready-to-load and does not require any additions or heating. It needs to be fully thawed and thoroughly mixed prior to using. Avoid vigorous vortexing, as buffers contain detergent. Following mixing, briefly pulse in a microcentrifuge to collect material from cap.

- Background
- Background: Alternative oxidases (**AOX**) are quinol oxidases located in the inner mitochondrial membrane of plants. They function as terminal oxidases in the alternate electron transport pathway, oxidizing ubiquinone to reduce oxygen to water.

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Source of AOX standard: AOX standard source is *Sphingomonas wittichii* strain RW1, overexpressed in E.coli bearing an N-terminal his6 tag.

- Product Citations
- Selected references: 38221900