

General protein extraction and immunoblotting for phytoplankton and plants

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Extraction Buffer:

A single extraction buffer is used for a single step cell disruption and solubilization of proteins from all phytoplankton and plant species.

Samples are suspended in:

140mM Tris base

105mM Tris-HCl

0.5mM ethylenediaminetetraacetic acid (EDTA)

2% Lithium dodecyl sulfate (LDS)

10% glycerol

0.1mg/mL PefaBloc SC (AEBSF) protease inhibitor (Roche).

We make a 4X extraction buffer with the LDS, Tris, Trizma, EDTA, glycerol (above concentrations are 1X), which can be stored at room temp. A 50X PefaBloc solution (5mg/ml) is made separately in 1X sample buffer and stored separately at -20C

Extraction Procedure:

To limit sample volumes to experimentally practical levels for sonication with a microtip, cell pellets in 2mL conical-bottom polypropylene tubes are resuspended in 150-350 μ l extraction buffer, depending on desired final chl (0.01-0.05mg/mL) or protein (0.1-0.5mg/mL) concentrations and the starting cell contents of the samples.

Cells initially harvested onto Whatman GFF filters can be extracted using direct sonication by saturating the filter with extraction buffer, but if cell numbers and extraction buffer volume are low, the glass powder can interfere with later recuperation of the protein extract from the tube.

If cells are harvested onto GFF, 'vacuum' as much of the source water/media from the filter as possible to minimize carryover into the extraction buffer. This is particularly important for seawater, which can interfere with extraction components and subsequent gel migration.

If using cells harvested onto nitrocellulose or PVDF filters, you will need to transfer the cells quantitatively to a 1.5 ml Ependorf tube.

If cell numbers are known or other data has been collected for the cultures on a per ml basis, be very careful to know the transfer volume in order to normalize to the original culture data.

You can either rinse cells into tubes using ddH₂O and then add an appropriate volume of 4X extraction buffer, or you can try just transferring them directly using 1X transfer buffer. If cells have been frozen (for example, after harvest onto a filter) upon thawing some cell content will leak out. Therefore, if transferring cells from a membrane to a tube for sonication, do not to perform washes (ex. do not transfer using a volume of water, then pellet the cells, then resuspend into a volume of extraction buffer; some proteins will be lost into the initial water volume. The risk is differential extraction of proteins during the cell transfer, with recalcitrant proteins left behind with debris on the filter, and soluble proteins transferred over more completely to the tube.

Plant tissue is weighed and snap frozen in liquid nitrogen. The frozen tissue is ground in liquid nitrogen in a pre-chilled mortar and pestle. The fine frozen powder is transferred to a 1.5 mL Eppendorf tube and extraction buffer is added (10 uL per mg wet weight often works well).

Cell samples suspended in extraction buffer are immediately frozen in liquid nitrogen and then sonicated with a microtip attachment at a setting of 30%, until just thawed.

To avoid heating, samples are then refrozen immediately in liquid N₂.

In general 2-3 rounds of freezing/sonication results in maximum extraction of protein, and further rounds are not beneficial, with risk of fragmentation of some proteins.

In trials we achieve 85-90% total protein extraction with 2-3 rounds, across a range of phytoplankton species.

Excessive heating during sonication leads to protein degradation and cleavage - keeping samples cold is important.

Following disruption, samples are centrifuged for 3 min at 10000 x g to remove insoluble material and any unbroken cells.

Remaining color in the pellet is a good indicator of incomplete breakage.

The protein content of the extracts is determined using BioRad DC Protein Assay (Modified Lowry) using Bovine Gamma Globulin in Extraction Buffer as a standard. It is important that the protein standard and the cell extract are in the same extraction buffer for reliable quantitation.

Sample Loading:

Samples in extraction buffer (see above) are brought to 50 mM dithiothreitol (DTT) final concentration by adding 1/10th final volume of 0.5 M DTT.

Cellular extracts are then heated at 70°C for 5 min, and recombinant protein standards are heated at 95°C for 5 min.

Excessive heating of whole cell extracts causes aggregation of some protein complexes, particularly membrane complexes.

Following heating, samples are pulsed briefly in a microcentrifuge to collect all of the material at the bottom of the tube.

Loading based on total protein quantitations gives more consistent results than loading on other bases (for example chlorophyll often changes during treatments).

Approximate gel loads: 0.5-2.5 ug total protein per lane.

As described above, our samples are suspended at approximately 0.1 µg total protein per µl, so our total loads are in the range of 10 µl.

For best quantitation we keep the sample total load volume as consistent as practical, because variable loading volumes can cause distortions in the migration and quantitation. Note that for quantitation we do not overload the gel/immunoblot. Heavily loaded gels give attractive images, but can deplete the antibody from solution and result in poor quantitations, especially when using ECL Advance chemiluminescent reagent.

Immunoblotting and quantitation:

Proteins are separated by electrophoresis on 4-12% acrylamide gradient mini-gels (NuPAGE Bis-Tris gels, Invitrogen) in MES or MOPS SDS running buffer (Invitrogen) in an XCell Sure-Lock Tank (Invitrogen).

Gels are electrophoresed at 200V for 35-60 minutes, with shorter times for smaller proteins.

Following electrophoresis the proteins are transferred to polyvinylidene difluoride (PVDF) membranes pre-wetted in methanol and equilibrated in 1X transfer buffer (Invitrogen) using the XCell blot module (Invitrogen) for 60 to 80 minutes at 30V, depending on protein size and the number of transfers per cell.

Blots are blocked immediately following transfer in 2% ECL Advance blocking reagent (GE Healthcare) in 20 mM Tris, 137 mM sodium chloride pH 7.6 with 0.1%(v/v) Tween-20 (TBS-T) for 1h at room temperature with agitation or overnight at 4°C.

Primary and secondary antibodies are used at a dilution of 1:20,000 to 1:50,000 in 2% ECL Advance Blocking solution.

Blots are incubated in the primary antibody solution for 1h at room temperature with agitation. The antibody solution is decanted and the blot is rinsed briefly twice, then washed once for 15 min and 3 times for 5 min in TBS-T at room temperature with agitation.

Blots are incubated in secondary antibody (either rabbit anti-chicken horse radish peroxidase conjugated or goat anti-rabbit horse radish peroxidase conjugated, both from AbCAM) diluted to 1:50,000 in 2% ECL Advance blocking solution for 1h at room temperature with agitation.

It is important the blot tray or box has a smooth bottom to avoid scraping the blot membrane, which results in variable background.

The blots are washed as above and developed for 5 min with ECL Advance detection reagent according the manufacturer's instructions (3 ml reagent per 68 x 81 mm blot). Images of the blots are obtained using a CCD imager (FluorSMax, Bio-Rad) and Quantity One software (Bio-Rad).

Other electrophoresis and immunoblotting protocols can generate good quality images, but in our experience the InvitrogenNuPAGE system is the most reliable for immunoquantitation of a wide range of soluble and membrane proteins.

Protein levels on immunoblots are quantitated using Quantity One software (Bio-Rad). We typically achieve a 15X range of pseudo-linear load/signal response on a single immunoblot, so all samples to be quantified on a given blot must contain the target protein within this 15X range. A wider overall dynamic range for quantification can be achieved across multiple blots by varying sample loads, antibody dilutions and detection times.

The absolute range of protein detection is much wider than 15X, but there are typically plateau regions at low and high target protein loads.