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A Protocol for the Identification of Protein-protein Interactions Based on ^{15}N Metabolic Labeling, Immunoprecipitation, Quantitative Mass Spectrometry and Affinity Modulation

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Abstract

Protein-protein interactions are fundamental for many biological processes in the cell. Therefore, their characterization plays an important role in current research and a plethora of methods for their investigation is available¹. Protein-protein interactions often are highly dynamic and may depend on subcellular localization, post-translational modifications and the local protein environment². Therefore, they should be investigated in their natural environment, for which co-immunoprecipitation approaches are the method of choice³. Co-precipitated interaction partners are identified either by immunoblotting in a targeted approach, or by mass spectrometry (LC-MS/MS) in an untargeted way. The latter strategy often is adversely affected by a large number of false positive discoveries, mainly derived from the high sensitivity of modern mass spectrometers that confidently detect traces of unspecifically precipitating proteins. A recent approach to overcome this problem is based on the idea that reduced amounts of specific interaction partners will co-precipitate with a given target protein whose cellular concentration is reduced by RNAi, while the amounts of unspecifically precipitating proteins should be unaffected. This approach, termed QUICK for QUAntitative Immunoprecipitation Combined with Knockdown⁴, employs Stable Isotope Labeling of Amino acids in Cell culture (SILAC)⁵ and MS to quantify the amounts of proteins immunoprecipitated from wild-type and knock-down strains. Proteins found in a 1:1 ratio can be considered as contaminants, those enriched in precipitates from the wild type as specific interaction partners of the target protein. Although innovative, QUICK bears some limitations: first, SILAC is

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cost-intensive and limited to organisms that ideally are auxotrophic for arginine and/or lysine. Moreover, when heavy arginine is fed, arginine-to-proline interconversion results in additional mass shifts for each proline in a peptide and slightly dilutes heavy with light arginine, which makes quantification more tedious and less accurate^{5,6}. Second, QUICK requires that antibodies are titrated such that they do not become saturated with target protein in extracts from knock-down mutants.

Here we introduce a modified QUICK protocol which overcomes the abovementioned limitations of QUICK by replacing SILAC for ¹⁵N metabolic labeling and by replacing RNAi-mediated knock-down for affinity modulation of protein-protein interactions. We demonstrate the applicability of this protocol using the unicellular green alga *Chlamydomonas reinhardtii* as model organism and the chloroplast HSP70B chaperone as target protein⁷ (**Figure 1**). HSP70s are known to interact with specific co-chaperones and substrates only in the ADP state⁸. We exploit this property as a means to verify the specific interaction of HSP70B with its nucleotide exchange factor CGE1⁹.



Protocol

1. Antibody Adsorption

1. Weigh out 120 mg of Protein A Sepharose in a 15-ml conical tube (Falcon). As 15 mg Protein A Sepharose is needed for each immunoprecipitation (IP) this amount is sufficient for 8 IPs. Add 5 ml 0.1 M phosphate buffer (pH 7.4) and let the Protein A Sepharose swell for 30 min at 4 °C.

(Note that all steps from this point on need to be carried out with gloves to avoid contamination with keratin and on ice to avoid protein degradation/complex dissociation.)

1. Centrifuge for 60 sec at 1,000 x g and 4 °C to pellet the swollen Protein A Sepharose. Carefully remove the supernatant and resuspend the beads in 5 ml 0.1 M phosphate buffer (pH 7.4). Repeat this step three times to wash the beads thoroughly.
2. After the last centrifugation step, remove supernatant and leave approximately 0.5 ml of phosphate buffer. Add 0.9 ml 0.5 M phosphate buffer (pH 7.4), 400 µl affinity purified primary antibodies (50 µl per IP) against the target protein (here HSP70B), and 16 µl antibodies against a control protein (here CF1β). Fill up with ddH₂O to a total volume of 5 ml.

(Note that affinity-purified antibodies should be used to reduce contamination by unspecific IgGs, which interfere with nano-LC-

MS analysis - for a protocol see Willmund et al. (2005)¹⁰. CF1β is precipitated as a loading control and was chosen because it is abundant and, after cell lysis, present in soluble and membrane fractions. Alternatively, levels of contaminating proteins may be used to normalize for unequal loading.)

1. Allow Protein A Sepharose beads to adsorb IgGs during a 1-hr incubation at 25 °C on a tube roller (CAT RM5W, 36 rpm).
2. Centrifuge for 60 sec at 1,000 x g and 4 °C to pellet the Protein A Sepharose beads. Carefully remove the supernatant and resuspend the beads in 5 ml 0.1 M sodium borate buffer (pH 9.0). Repeat this step three times to thoroughly remove amines that would quench the crosslinker.
3. Weigh out 25.9 mg of fresh, solid dimethylpimelimidate and resuspend it in 5 ml 0.1 M sodium borate buffer (pH 9.0) to obtain a final concentration of 20 mM. Add this solution to the Protein A Sepharose beads.
4. Allow IgGs to cross-link to Protein A for 30 min at 25 °C on a tube roller.
5. Centrifuge for 60 sec at 1,000 x g and 4 °C to pellet the beads. Carefully remove the supernatant and resuspend the beads in 5 ml 1 M Tris-HCl (pH 7.5) to quench free crosslinker. Repeat this step once and incubate for 2 hr at 25 °C or 12-24 hr at 4 °C on a tube roller.
6. Optional: if Protein A Sepharose beads coupled to IgGs are not directly used for IP, storage for up to one week is possible. For this, centrifuge for 60 sec at 1,000 x g and 4 °C to pellet the beads, carefully remove the supernatant and resuspend beads in 5 ml 0.1 M phosphate buffer (pH 7.5) containing 0.02% sodium azide and store at 4 °C until further use.

2. Cell Lysis, Crosslinking and Sample Preparation

1. Grow two Chlamydomonas cultures in medium containing 7.5 mM ¹⁴NH₄Cl or ¹⁵NH₄Cl as nitrogen source to a density of ~5 x 10⁶ cells/ml. Cells need to pass through at least ten generations for full labeling. Here, cells were grown photomixotrophically in TAP medium¹¹ on a rotatory shaker at 25 °C under continuous irradiation with white light (30 μE m⁻² s⁻¹).
2. Transfer two aliquots each of ¹⁴N- and ¹⁵N-labeled cells to four GSA tubes and harvest cells by a 4-min centrifugation at 4,000 x g and 4 °C (*The cell number harvested for each aliquot depends on the cellular concentration of the target protein and needs to be determined empirically in advance to ensure that sufficient target protein is precipitated. A good starting point is 10⁹ cells per aliquot, i.e., 200 ml of a culture with 5 x 10⁶ cells/ml.*)**For crosslinking only:** in case protein complexes will be crosslinked prior to IP, cells need to be washed to remove amines present in the medium. For this, resuspend cells in 40 ml pre-cooled KH buffer (20 mM HEPES-KOH (pH 7.2), 80 mM KCl) and transfer them to 50-ml Falcon tubes. Centrifuge for 60 sec at 1,000 x g and 4 °C. Repeat this step once.
3. Resuspend cells in 2 ml Lysis buffer (20 mM HEPES-KOH (pH 7.2), 1 mM MgCl₂, 10 mM KCl, 154 mM NaCl) pre-cooled to 4 °C and

transfer them to 15-ml Falcon tubes. Collect remaining cells in GSA tubes with an additional 1 ml Lysis buffer each. Add 50 μ l 25 x protease inhibitor and 12.5 μ l 1 M MgCl₂ (to a final concentration of 3.5 mM) to each aliquot.

4. Add 150 μ l Lysis buffer, 12.5 μ l 1 M ATP, 833 μ l 270 mM creatine phosphate and 7 μ l 5 μ g/ μ l creatine phosphokinase (the final concentration is 2.5 mM ATP, 45 mM creatine phosphate, and 7 μ g/ml creatine phosphokinase) to one of the aliquots containing ¹⁴N- and ¹⁵N-labeled cells (these are the +ATP aliquots).
5. Add 930 μ l Lysis buffer and 70 μ l 1 U/ μ l apyrase to the other aliquots containing ¹⁴N- and ¹⁵N-labeled cells (these are the -ATP aliquots).
6. Incubate for 2 min at 25 °C on a tube roller to establish ATP-deplete and ATP-replete states. If the crosslinking step is omitted, add another 1 ml of Lysis buffer. **For crosslinking only:** in case the investigated protein-protein interactions are transient it is advisable to capture them by a crosslinking step. For this, add 500 μ l 20 mM dithio-bis(succinimidyl propionate) (DSP) dissolved in DMSO (final concentration is 2 mM) to each tube directly before sonication.
7. Sonicate four times 20 sec on ice to break cells with 20-sec breaks in between for cooling. (We use the Bandelin Sonoplus HD2070 with a KE76 tip at output control of 75% and duty cycle of 60%. The necessary settings for other machines/devices/tips need to be determined in advance to ensure complete cell lysis and to avoid spilling.) **For crosslinking only:** allow protein complexes to crosslink by incubating for 1 hr at 4 °C on a tube roller. After crosslinking, supplement each tube with 500 μ l 1 M glycine and incubate on a tube roller for another 15 min at 4 °C to quench free crosslinker.
8. Prepare four 6-ml sucrose cushions (20 mM HEPES-KOH (pH 7.2), 0.6 M sucrose) in SW41 Ti thin wall tubes (Beckman Coulter Item No: 344059), carefully lay the entire ~5.5 ml of cell lysates onto the sucrose cushions (balance with Lysis buffer) and centrifuge for 30 min at 200,000 x g and 4 °C in a SW41 Ti rotor.
9. Transfer the top of the gradient containing soluble protein complexes into four 15-ml Falcon tubes (avoid transferring parts of the sucrose cushion), add 350 μ l 10% Triton X-100 to a final concentration of 0.5% to each of them, mix carefully and add Lysis buffer to a total volume of 7 ml each. (*Transfer 70 μ l of each soluble cell extract to fresh 1.5-ml conical tubes (Eppendorf tubes) and add 70 μ l 2 x SDS-sample buffer (4% SDS, 125 mM Tris-HCl (pH 6.8), 20% glycerol, 10% 2-mercaptoethanol) to each for SDS-PAGE and immunoblot analyses.*)
10. Discard the sucrose cushions and resuspend the membrane pellets in 3 ml Lysis buffer each. Add to each 1 ml 10% Triton X-100 to a final concentration of 2%, sonicate on ice to dissolve pellets, and add Lysis buffer to a total volume of 5 ml each.
11. Prepare another four 6-ml sucrose cushions in SW41 Ti thin wall tubes, lay the ~5 ml of solubilized membranes from step 2.10 carefully onto the sucrose cushions, and centrifuge for 30 min at 200,000 x g and 4 °C in a SW41 Ti rotor.
12. Transfer the top of the gradient containing membrane protein complexes into four 15-ml Falcon tubes and add Lysis buffer

(containing 2% Triton X-100) to a final volume of 7 ml each. *(Transfer 70 µl of each soluble cell extract to fresh Eppendorf tubes and add 70 µl 2 x SDS-sample to each for SDS-PAGE and immunoblot analyses.)*

3. Immunoprecipitation

1. Pellet the Protein A Sepharose beads containing coupled antibodies (from steps 1.8 or 1.9) by a 60-sec centrifugation at 1,000 x g and 4 °C, carefully remove the supernatant and resuspend beads in 4 ml Lysis buffer. Repeat this step twice to equilibrate beads in Lysis buffer.
2. Fill up to 8 ml with Lysis buffer and transfer 1 ml of the suspension to each of the eight 15-ml Falcon tubes containing soluble or membrane protein complexes from ATP-replete and ATP-deplete ¹⁴N- and ¹⁵N-labeled cells (from step 2.9 and 2.12).
3. Incubate for 2 hr at 4 °C on a tube roller to precipitate protein complexes.
4. Pellet the beads by a 60-sec centrifugation at 1,000 x g and 4 °C and discard the supernatants. Leave a small volume of liquid on top of the beads to facilitate transfer.
5. Transfer the beads from each Falcon tube to 1.5-ml conical tubes (Eppendorf tubes). To collect all remaining beads in the Falcon tubes, add another 0.8 ml Lysis buffer containing 0.1% Triton to each, vortex gently, centrifuge for 60 sec at 1,000 x g and 4 °C and transfer the buffer with residual beads to the Eppendorf tubes. Tube exchange is necessary to prevent contaminations from proteins adhering to the plastic walls.
6. Pellet the beads by a 15-sec centrifugation at 16,100 x g and 4 °C, carefully remove the supernatants and resuspend beads in 1.3 ml Lysis buffer containing 0.1% Triton. Repeat this step twice with lysis buffer containing Triton and twice with Lysis buffer lacking Triton to thoroughly wash the beads. Leave a small volume of liquid on top of the beads to facilitate transfer.
7. Again transfer the beads to fresh 1.5-ml Eppendorf tubes to remove proteins adhering to the plastic walls. Wash the old tubes with 1 ml Lysis buffer lacking Triton and transfer all residual beads to the fresh tubes.
8. Centrifuge for 15 sec at 16,100 x g and 4 °C, remove the supernatants first with a normal pipette, then remove any remaining supernatant completely with a 50-µl Hamilton syringe.

4. Sample Preparation for nano-LC-MS/MS

1. Add 100 µl freshly prepared Elution buffer (8 M urea, 25 mM NH₄HCO₃) to each tube, use the 100 µl Elution buffer to wash off beads sticking to the Hamilton syringe and incubate for 10 min in a thermomixer at 800 rpm and 65 °C, and for another 20 min at 30 °C. *(A much more complete elution of bound proteins is achieved by elution with 2% SDS and subsequent precipitation with 80% acetone.)*

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2. Centrifuge for 15 sec at 16,100 x g and 25 °C. Transfer supernatants to fresh tubes with a 50- μ l Hamilton syringe.
3. Add 50 μ l Elution buffer to the beads, use the 50 μ l Elution buffer to wash off beads sticking to Hamilton syringe and repeat incubation and centrifugation steps 4.1 and 4.2, respectively. Pool the respective eluates. (*Transfer 30 μ l of the eluates to fresh Eppendorf tubes, add 30 μ l 2 x SDS-sample buffer to each for SDS-PAGE and immunoblot analyses.*)
4. Combine the eluted precipitates from +/-ATP-treated, ¹⁴N- and ¹⁵N-labeled soluble and membrane proteins as follows: 120 μ l ¹⁵N/+ATP and 120 μ l ¹⁴N/-ATP 120 μ l ¹⁴N/+ATP and 120 μ l ¹⁵N/-ATP 120 μ l ¹⁵N/+ATP and 120 μ l ¹⁴N/-ATP 120 μ l ¹⁴N/+ATP and 120 μ l ¹⁵N/-ATP
5. Add 1.5 μ l freshly prepared 1 M DTT to a final concentration of 6.5 mM to each of the four combinations to reduce disulfide bonds (including those in the crosslinker) and incubate for 30 min at 25 °C.
6. Add 10.5 μ l freshly prepared 0.6 M iodoacetamide to a final concentration of 25 mM to carboxymethylate the reduced thiols and incubate for 20 min at 25 °C in the dark.
7. Add 256 μ l 40 mM NH₄HCO₃ and 4 μ l Lys-C (0.1 μ g/ μ l), seal tubes with parafilm, and incubate for at least 16 hr overnight on a rotation wheel at 37 °C.
8. Add 470 μ l 20 mM NH₄HCO₃, 10 μ l 100 % acetonitrile (to a final concentration of 1%) and 8 μ l trypsin beads, and incubate on a rotation wheel for at least 16 hr at 37 °C.
9. Centrifuge for 5 min at 16,100 x g and 4 °C, and transfer supernatants to fresh 2-ml Eppendorf tubes. Wash the old tubes with 50 μ l 20 mM NH₄HCO₃, 0.5% acetic acid, and pool with first supernatants.
10. For desalting, prepare homemade C₁₈-StageTips by cutting out two discs from Empore C₁₈ material with a syringe needle and placing them in a 200- μ l pipette tip. In this way prepare four 200- μ l tips. Punch holes into the lids of four 2-ml Eppendorf tubes and insert tips.
11. Precondition the C₁₈-StageTips with 50 μ l Solution B (80% acetonitrile, 0.5% acetic acid). Centrifuge for 3 min at 800 g and 25 °C.
12. Equilibrate the C₁₈-StageTips with 100 μ l Solution A (0.5% acetic acid, 2% acetonitrile). Centrifuge for 3 min at 800 g and 25 °C. Repeat this step once.
13. Load 100 μ l of the supernatants from the tryptic digestions (4.9) on the C₁₈-StageTips and centrifuge for 3 min at 800 g and 25 °C. Repeat this step until the complete supernatants were applied to the columns.
14. Wash the C₁₈-StageTips with 100 μ l Solution A. Centrifuge for 3 min at 800 g and 25 °C. Repeat this step twice.
15. Elute tryptic peptides into a fresh 1.5-ml Eppendorf tube with 50 μ l Solution B. Centrifuge for 3 min at 800 g and 25 °C. Repeat this step once. Dry peptides to completion in a speed vac.

16. Optional: seal Eppendorf tubes with parafilm and store at -80 °C until further use.
17. Resuspend the dried peptides with 20 µl Solution A and incubate for at least 1 hr on ice, interrupted by two 15-min incubations in a sonicator bath. Centrifuge for 20 min at 16,100 x g and 4 °C, and apply supernatant to nano-LC-MS/MS.

5. Representative Results

As shown exemplarily for the ¹⁴N-labeled cell extracts in **Figure 2A**, HSP70B and CGE1 are almost exclusively localized to the soluble fraction, independent of the ATP state. In contrast, CF1β is localized to soluble and membrane-enriched fractions, as sonication shears part of it from membrane-located CF_o, and therefore serves as loading control for both fractions. As shown in **Figure 2B**, similar amounts of HSP70B were precipitated with the anti-HSP70B antibodies from ¹⁴N- and ¹⁵N-labeled soluble extracts, independent of the ATP state. In contrast, only little HSP70B was precipitated from membrane fractions with slightly larger amounts originating from ATP-depleted membrane fractions as compared to ATP replete fractions, hence corroborating earlier results⁹. No CGE1 was co-precipitated with HSP70B in ATP-replete soluble or membrane fractions, while large amounts of CGE1 were co-precipitated with HSP70B from ATP-depleted soluble fractions, and little from ATP-depleted membrane fractions.

The interaction of CGE1 with HSP70B only in the ADP state is also observed in the MS analysis: in **Figure 3**, representative MS1 spectra of HSP70B and CGE1 peptides from precipitates generated with the HSP70B antiserum from soluble cell extracts are shown. In the experiment shown in **Figure 3A**, precipitates were from mixtures of ¹⁴N-labeled extracts lacking ATP and ¹⁵N-labeled extracts containing ATP. While the heavy and light labeled form of the HSP70B peptide were detected at equal intensities, only the light labeled form of the CGE1 peptide (from -ATP extracts) was found. In **Figure 3B** the same peptides from the anti-HSP70B precipitate derived from mixtures of reciprocally labeled soluble cell extracts are shown. Accordingly, this time only the heavy labeled form of the CGE1 peptide (from -ATP extracts) was detected, while this was again the case for both, light and heavy labeled HSP70B peptides.

Figure 1. Experimental workflow. Cells are metabolically labeled with ¹⁴N and ¹⁵N for at least 10 generations, harvested and supplied with or depleted from ATP. After cell lysis protein complexes optionally may be crosslinked (X-link) with DSP. Lysed cells are then separated in soluble (Sol) and membrane enriched (Pel) fractions. Target proteins (here HSP70B) and a control protein (here CF1β) are immunoprecipitated with specific antibodies coupled to protein A sepharose beads (black). After washing, precipitated proteins are eluted and either directly analyzed by immunoblotting, or the respective ¹⁴N- and ¹⁵N-labeled fractions in +ATP and -ATP states are pooled, digested and analyzed by nano-LC-MS/MS. In the example case shown here the ¹⁵N-labeled fraction was depleted from ATP. Accordingly, the ratio of intensities of heavy labeled (dark colors) to light labeled (light colors) peptides from the control protein (CF1β), the target protein (HSP70B) and non-specifically bound

contaminants should be around one, while this ratio is expected to be very high for proteins specifically interacting with the target protein (CGE1). [Click here to view larger figure.](#)

Figure 2. A Analysis of the input for HSP70B

immunoprecipitation. Total protein was extracted from soluble (Sol) and membrane enriched (Pel) fractions either depleted from ATP (-ATP) or supplemented with ATP and an ATP regenerating system (+ATP). 0.01% of the protein extracts were separated on a 10% SDS-polyacrylamide gel, and levels of HSP70B and CGE1 protein relative to loading control CF1 β were analyzed by immunoblotting. **B Analysis of immunoprecipitates.** HSP70B was immunoprecipitated from ¹⁴N- and ¹⁵N-labeled soluble and membrane-enriched cell extracts containing or lacking ATP. Proteins corresponding to 3.3% of the immunoprecipitates were separated on a 10% SDS-polyacrylamide gel and levels of HSP70B and CGE1 relative to loading control CF1 β were analyzed by immunoblotting. [Click here to view larger figure.](#)

Figure 3. A Representative mass spectra of HSP70B and CGE1 peptides from anti-HSP70B immunoprecipitates performed on mixed soluble fractions (¹⁴N -ATP/¹⁵N +ATP). Full MS spectra of ¹⁴N and ¹⁵N labeled peptides, corresponding to the -ATP and +ATP states, respectively, from HSP70B and co-immunoprecipitated CGE1 are shown. Both peptides are triply charged, the HSP70B peptide contains 22 nitrogen atoms, the CGE1 peptide 19, corresponding to a mass shift of 7.33 and 6.33 m/z, respectively. **B Representative mass spectra from the reciprocal experiment (¹⁴N +ATP/¹⁵N -ATP).** Full MS spectra of the same ¹⁴N and ¹⁵N labeled peptides, here corresponding to the +ATP and -ATP states, respectively, from HSP70B and co-immunoprecipitated CGE1 are shown. [Click here to view larger figure.](#)

Discussion

We have recently introduced two improvements to the QUICK approach: a crosslinking step for capturing transient protein-protein interactions (QUICK-X), and a control precipitation to normalize for unequal precipitation efficiencies⁶. Here we present a protocol containing two more improvements of QUICK: first, we replace SILAC⁵ for ¹⁵N metabolic labeling. The advantages are that ¹⁵N metabolic labeling is much cheaper than SILAC, if ¹⁵N is provided as simple inorganic salt. Furthermore, with ¹⁵N metabolic labeling QUICK can be applied to organisms prototrophic for all amino acids, like most plants, fungi and bacteria. And finally, arginine-to-proline interconversion inherent to SILAC^{5,6} does not present a problem for quantification of ¹⁵N labeled peptides. Examples for suitable tools for the quantitative evaluation of ¹⁵N proteomics data are MSQUANT¹² or IOMIQS¹³.

Second, we introduce affinity modulation as a means for specifically reducing the amount of proteins interacting with a given target protein in one sample versus another. The advantages of this approach are that it circumvents the construction of knockdown mutants, which for some model systems are difficult to generate or cannot be generated at all in case of essential target proteins. Moreover, it avoids misinterpretations caused by differential protein expression potentially occurring as a

response of the cell to knocking-down a target protein: if other proteins are down-regulated as well and cross-react with the antiserum used for immunoprecipitation, they would be interpreted as true interaction partners of the target protein. At last, affinity modulation abolishes the need of finding a proper antibody-to-antigen ratio.

Although we apply our protocol to *Chlamydomonas reinhardtii* as model organism, it can easily be adapted to any other organism that can be grown in cell culture and is able to use ammonium or nitrate as nitrogen source. Affinity modulation of protein complexes by ATP/ADP may directly be applied to other chaperones whose interaction with substrates and cohort proteins depends on the ATP state, like the GroEL/HSP60/Cpn60 or HSP90 chaperone systems^{14,15}, or to any other system where binding affinities are modulated by ATP. Affinity modulation should also work for cases where affinities between protein interactions are altered by specific drugs, like radicicol or geldanamycin in the case of HSP90 systems¹⁵.

A clear limitation of our protocol is that it requires affinity-purified antibodies against a target protein known to be sensitive to a specific treatment/drug that modulates its affinity for partner proteins. Therefore, it is no high-throughput method.

Disclosures

No conflicts of interest declared.

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References

- 1 Perrakis A Musacchio A Cusack S Petosa C Investigating a macromolecular complex: the toolkit of methods J. Struct. Biol 2011 175 106 112 21620973
- 2 Gavin AC Maeda K Kuhner S Recent advances in charting protein-protein interaction: mass spectrometry-based approaches Curr. Opin. Biotechnol 2011 22 42 49 20934865
- 3 Markham K Bai Y Schmitt-Ulms G Co-immunoprecipitations revisited: an update on experimental concepts and their implementation for sensitive interactome investigations of endogenous proteins Anal. Bioanal. Chem 2007 389 461 473 17583802
- 4 Selbach M Mann M Protein interaction screening by quantitative immunoprecipitation combined with knockdown (QUICK Nat Methods 2006 3 981 983 17072306
- 5 Ong SE Mann M A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC) Nat Protoc 2006 1 2650 2660 17406521
- 6 Heide H Application of quantitative immunoprecipitation combined with knockdown and cross-linking to *Chlamydomonas* reveals the presence of vesicle-inducing protein in plastids 1 in a common complex with chloroplast HSP90C Proteomics 2009 9 3079 3089 19526558

- 10 A Protocol for the Identification of Protein-protein Interactions Based on ¹⁵N Metabolic Labeling, Immunoprecipitation, Quantitative Mass Spectrometry and Affinity Modulation
- 7 Nordhues A Miller SM Muhlhaus T Schroda M New insights into the roles of molecular chaperones in Chlamydomonas and Volvox International review of cell and molecular biology 2010 285 75 113 21035098
- 8 Mayer MP Bukau B Hsp70 chaperones: cellular functions and molecular mechanism Cell Mol. Life Sci 2005 62 670 684 15770419
- 9 Schroda M Vallon O Whitelegge JP Beck CF Wollman FA The chloroplastic GrpE homolog of Chlamydomonas: two isoforms generated by differential splicing The Plant Cell 2001 13 2823 2839 11752390
- 10 Willmund F Schroda M HEAT SHOCK PROTEIN 90C is a bona fide Hsp90 that interacts with plastidic HSP70B in Chlamydomonas reinhardtii Plant Physiol 2005 138 2310 2322 15995001
- 11 Harris EH The Chlamydomonas Sourcebook: Introduction to Chlamydomonas and Its Laboratory Use 2008 San Diego, CA Elsevier, Academic Press
- 12 Mortensen P MSQuant, an open source platform for mass spectrometry-based quantitative proteomics J. Proteome Res 2010 9 393 403 19888749
- 13 Muhlhaus T Weiss J Hemme D Sommer F Schroda M Quantitative Shotgun Proteomics Using a Uniform ¹⁵N-Labeled Standard to Monitor Proteome Dynamics in Time Course Experiments Reveals New Insights into the Heat Stress Response of Chlamydomonas reinhardtii Mol. Cell Proteomics 2011 10
- 14 Bukau B Horwich AL The Hsp70 and Hsp60 chaperone machines Cell 1998 92 351 366 9476895
- 15 Wandinger SK Richter K Buchner J The Hsp90 chaperone machinery J. Biol. Chem 2008 283 18473 18477 18442971