HA tag enables highly efficient detection of heterologous proteins in Phaeodactylum tricornutum (Pt) exconjugants

Version 2

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Abstract

Invitrogen's HA tag monoclonal antibody in combination with Western Breeze Anti-Mouse Chemiluminescent Western Blot Immunodetection Kit leads to no background Western blots with Pt lysates. HA tag thus represents a tag of choice for (1) efficient and unambioguous protein expression analysis, (2) immunofluorescence experiments and (3) various IP and pull-down experiments in Pt and possibly other diatoms as well as other marine microeukaryotes.

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Guidelines

Recommended HA tag nucleotide sequence is as follows: 5'-TATCCGTATGATGTCCCCGATTACGCG-3'.

Before start

The starting point of this protocol is a genotype+ genetically engineered *Pt* strain.

Protocol

Cell lysis

Step 1.

Spin down 10^7-10^8 *Pt* cells for 10 min at 4000 rpm & 10 °C.

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Keep tubes on ice throughout the protocol.

Step 2.

Resuspend pellets in 50-200 µL cell lysis buffer: 50 mM Tris-HCl, 200 mM NaCl, 1 mM DTT, 1 mM PMSF.

Step 3.

Sonicate as follows: 30 sec on / 1 min off for 15 min. We use Bioruptor UCD-200TM.

Step 4.

Separate supernatants and pellets/insoluble fractions by centrifuging lysates 45 min at 13500 rpm & 10 °C. Resuspend pellets/insoluble fractions in 100 µL lysis buffer.

Quantify total protein content in supernatants

Step 5.

Use your favorite protein quantification assay. We use ThermoFischer's Bradford assay.

Running SDS-PAGE

Step 6.

Prepare supernatants as follows (40 µL total): 10 µL NuPage LDS Sample Buffer (4x), 4 µL NuPAGE Sample Reducing Agent (10x), 40 µg protein, MQ.

Prepare pellets/insoluble fractions as follows (40 uL total): 10 µL NuPage LDS Sample Buffer (4x), 4 µL NuPAGE Sample Reducing Agent (10x), 4 µL pellet/insoluble fraction suspension, 22 µL MQ.

Incubate at 70 °C for 15 min.

Step 7.

Load 10 µL supernatant sample (10 µg protein) and 10 µL pellet/insoluble fraction sample (1 µL "stock"). Load also 6 µL MagicMark protein standard.

NOTES

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1. Prepare MOPS running buffer as follows: 665 mL MQ + 35 mL 20x MOPS buffer.

2. We have had good experience with NuPage 4-12% Bis-Tris 1.5 mm gels with 10 wells.

3. Add 500 µL NuPAGE Antioxidant to the inner reservoir.

Step 8.

Separate proteins for 50 min at 200 V.

Protein transfer

Step 9.

Prepare transfer buffer: 40 mL NuPage Transfer Buffer (20x), 160 mL methanol, 600 mL MQ, 800 µL NuPAGE Antioxidant.

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We do a wet transfer using XCell II™ Blot Module and PVDF membranes.

Step 10.

Soak 7 pads in transfer buffer.

Step 11.

Activate PVDF membranes in methanol (1 min is good). Wash in MQ, leave in transfer buffer.

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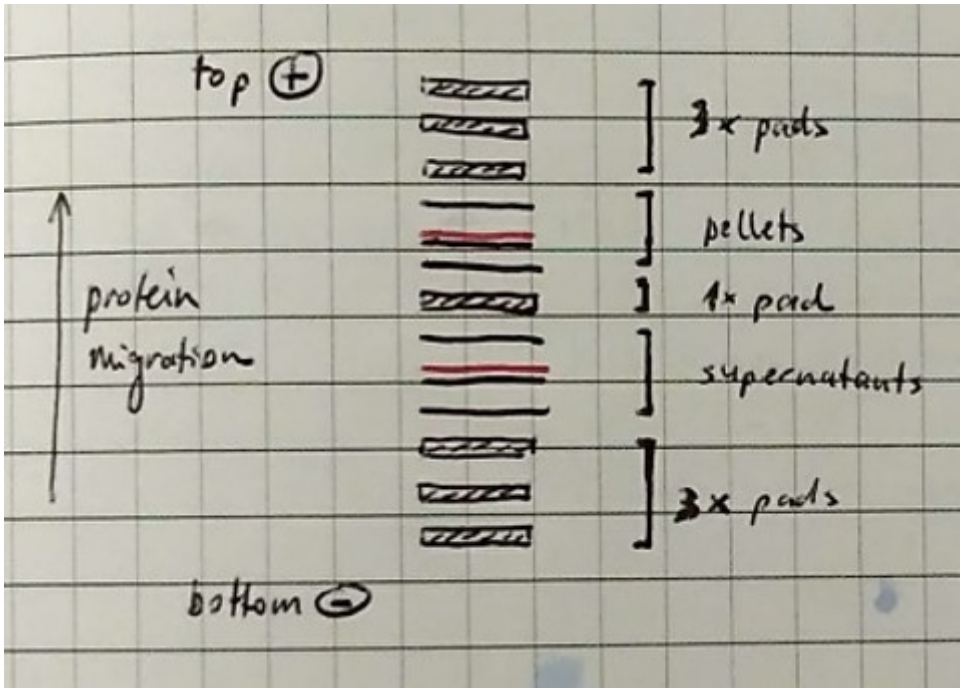
Leave filter papers that come with each membrane soaking in transfer buffer while you're activating membranes.

Step 12.

Dissassemble your gels and stack a transfer sandwich. If transferring from 2 gels simultaneously, we recommend assembling your sandwich as follows:



Step 13.

Transfer proteins for 1 h at 30 V.

Blocking

Step 14.

Cut top right corner of each membrane to track membrane faces containing proteins.

Step 15.

Wash each membrane twice with 20 mL MQ for 5 min.

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All washing and incubation steps at RT were performed on a rocking platform set to 1 (very gentle rocking).

Step 16.

Prapare blocking buffer. For 2 membranes: 10 mL MQ, 4 mL "Part A", 6 mL "Part B".

Step 17.

Block at least 1 h at room temperature. Use 10 mL/membrane.

NOTES

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The result in this protocol was obtained after a 2 day blocking step at 4 °C followed by another ~1 h 15 min RT incubation period.

Step 18.

Wash each membrane twice with 20 mL MQ for 5 min.

Incubation in primary antibodies

Step 19.

Prepare 1:10000 dilution of HA tag monoclonal antibody: 14 mL MQ, 4 mL "Part A", 2 mL "Part B", 2 µL antibody stock.

Step 20.

Use 10 mL antibody solution per membrane and incubate 3 hr.

Step 21.

Prepare 160 mL wash solution: 150 mL MQ + 10 mL Wash Solution (16x). Wash each membrane 4 times in 20 mL for 5 min.

Incubation in secondary antibodies

Step 22.

Incubate each membrane in 10 mL anti-mouse secondary antibody solution for 1 hr.

Step 23.

Prepare 160 mL wash solution: 150 mL MQ + 10 mL Wash Solution (16x). Wash each membrane 4 times in 20 mL for 5 min.

Step 24.

Wash each membrane 3 times in 20 mL MQ for 2 min.

Chemiluminescent detection

Step 25.

Remove excess liquid, place a membrane on transparent plastic foil and cover it evenly with 2 mL Novex CDP-Star alkaline phosphatase substrate.

Step 26.

Cover with alufoil and incubate 5 min.

Step 27.

Remove excess liquid and cover with another transparent plastic sheet.

Visualization

Step 28.

We use LI-COR's C-DiGit Blot Scanner.

Expected result

Step 29.

5 genotype+ *Pt* exconjugants were screened for protein-HA expression. The left and right image below were obtained with superntants and insoluble fractions, respectively.