Co-Immunoprecipitation (Co-IP) protocol for Nematostella vectensis v2.0

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**Homogenization Buffer (HB)**
Make fresh or one day before. Start with ~35 ml of DI water in a 50 ml Falcon tube. Add:
- 30mM HEPES, pH to 7.5
- 1mM EDTA
- 150mM NaCl
- 50mM NaF
- 1 mM Na3VO4
- 1% NP-40
- 10% Glycerol

fill to 50ml with DI H2O. This part will stay good for ~1 week at 4°C.

*Day of Use:* To 10 ml of homogenization buffer (in a 15ml Falcon tube), add:
- 50 µL Protease Inhibitor (Sigma P8340)
- 100 µL 10 mg/ml PMSF (in 100%EtOH; store at -20)

just before using. Only good ~24hrs.

**Washing Buffer (WB)**
Homogenization Buffer + NaCl up to 250mM

**Elution Buffer (AB)**
Use 2X Assayable Buffer
Make one day before. Start with ~3 ml of DI in a 15 ml Falcon tube. Add:
- 0.15 g Tris base; pH 6.8, add:
- 0.4 g SDS
- 1 ml of Glycerol

Fill to 10ml and allow to settle overnight. Store at RT (SDS comes out of solution in the fridge).

**Normal IgG**
Pierce Protein AG Magnetic Beads

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Salt concentration in HB is critical!!! 100mM < [Salt] < 150mM During IP NaCl or KCl. Here NaCl. Unspecific binding at low concentrations and no binding at higher concentrations.

Wash Buffer > [Salt]

KEEP AND DO EVERYTHING IN/ON ICE!!! DO IT QUICK!!! NEVER W/O SOLUTION!!!

**Homogenization**

1. Weigh tissue and record mass.
2. Suspend tissue in ice-cold homogenization buffer in sterile 1.5/2 ml eppendorf tube on ice (500 µl buffer/0.1 g embryos → ~500 µg/mL). Homogenize with a polytron or similar apparatus on high for 30s (homogenize 100%) on ice. This will give you solution H.
3. Incubate solution H for 30 minutes on ice

**Protein Isolation**
Centrifuge H at **max speed** (14,000 x g) for **30 min** at 4°C. This will give you solution S (supernatant); transfer (pour) S to 1.5 ml tube labeled S and centrifuge again at **max speed** (14,000 x g) for **10 min** at 4°C.

Remove **100 µl** of S and add it to **100 µl 2X assayable buffer** in 0.5 ml PCR tube labeled 2XAB. Save it on ice. Transfer all remaining supernatant from S to the 0.5 ml tube labeled **protein**; snap freeze protein samples on dry ice and store at -80 or keep it on ice if will use the same day.

**Protein Quantification**

1. Quantify total protein in your 2XAB samples with the **Pierce BCA protein assay** (23223). This assay is not sensitive to the concentration of SDS in the samples. **Make protein standards and dilutions with DI water, not buffer.**
2. Follow manufacturer’s protocol or see protocol: **Quantifying total protein with Pierce BCA.**
   
   **THE CONCENTRATION OBTAINED IS 0.5X OF YOUR TOTAL PROTEIN!!!!**
   
   **CONSIDER MAKE HIGHER DILUTIONS (5 TO 10 TIMES)**

**Co-IP**

**Day I**

**Antibody Crosslinking (capture the beads in every step! DO NOT LET THEM DRY!!!)**

1. Rinse **100µl** Protein A/G Magnetic Beads 3x with **1 mL** of PBS
2. Rinse **2x** with **1 mL** of **100mM Sodium Citrate, pH 5.0**
3. Dissolve the beads in **1mL** of Homogenization buffer and make **2 aliquots** of **500µL** of the resulting solution.
4. Add antibody of interest and Normal IgG to different aliquots: **5µg** of antibody (Ab-beads) and **5µg** of Normal IgG (not serum; IgG-beads), respectively.
5. Incubate by **2 hrs at RT with rotation**
6. Rinse **2x** with **1 mL** of **100mM Sodium Citrate, pH 5.0**
7. Rinse **1x** with **1 mL** of **200mM Triethanolamine, pH 8.2**
8. Prepare **20mM DMP** (Dimethyl pimelimidate dihydrochloride, D8388 SIGMA) solution in **200mM Triethanolamine, pH 8.2** (FRESH!!!)
9. Incubate the beads in **1 mL** of DMP solution by **45 min at RT with rotation** (crosslinking!!!)
10. Rinse **1x** with **1 mL** of **50mM Tris, pH 7.5**
11. Add **1 mL** of **50mM Tris, pH 7.5** and incubate by **45 min at RT with rotation** (STOP solution!!!)
12. Rinse **3x** with **PBTw** (PBS + 0.01% Tween20)
13. Add **1mL** of Homogenization buffer with **0.005% BSA** and incubate by **1 hr at RT with rotation** (Blocking!)

**Immunoprecipitation**

14. Take **1mg** of protein from your total protein extract and bring it to **500µl** with HB. (**Save at least 15-25µl to load as INPUT, can freeze, 4% protein**)
15. Add the Supernatant from step 14 (S1) to the IgG-beads and incubate by **1 hr at 4°C with rotation.**
16. Capture beads. Transfer supernatant (S2) to a new tube. Add WB to MB1. DO NOT LET THEM DRY!!! Wash 3x5 minutes at 4ºC with rotation.
17. Add S2 to Ab-beads and incubate at 4ºC with rotation O/N (IP/co-IP!!! solution)
18. Elute MB1 with 1x Sample buffer: add 15µl of 2xAB and pipette up/down, add 12.6µl of MiliQ water, BromoPhenol Blue (BPB) and Beta-MercaptoEthanol (BME) to final concentrations of 0.02% (1.2µl of 0.5% BPB for every 30µl sample) and 4% (1.2µl of BME for every 30µl of sample). Elute pipetting up & down several times (E-MB1).
19. Incubate E-MB1 at 80ºC for 10 minutes (in thermocycler).
20. Centrifuge E-MB1 at max speed (5 minutes) and collect supernatant (IgG GEL). Snap freeze and keep it until you load the gel.

Day II
21. Capture Ab-beads of IP solution. Transfer supernatant (S3) to a new tube. Add WB to IP. DO NOT LET THEM DRY!!! Wash 3x5 minutes at 4ºC with rotation.
22. Take 15-30µl of S3 and make 1x Sample buffer: 15µl of 2xAB, BromoPhenol Blue (BPB) and Beta-MercaptoEthanol (BME) to final concentrations of 0.02% (1.2µl of 0.5% BPB for every 30µl sample) and 4% (1.2µl of BME for every 30µl of sample) (S3 GEL). Snap freeze the rest of S3.
23. Incubate S3 GEL at 80ºC for 10 minutes (in thermocycler). Snap freeze and keep it until you load the gel.
24. Elute IP with 1x Sample buffer: add 15µl of 2xAB and pipette up/down, add 12.6µl of MiliQ water, add BromoPhenol Blue (BPB) and Beta-MercaptoEthanol (BME) to final concentrations of 0.02% (1.2µl of 0.5% BPB for every 30µl sample) and 4% (1.2µl of BME for every 30µl of sample). Elute pipetting up & down several times (E-IP).
25. Incubate E-IP at 80ºC for 10 minutes (in thermocycler).
26. Centrifuge E-IP at max speed (5 minutes) and collect supernatant (IP GEL). Snap freeze and keep it until you load the gel.

Day II or Day III (Western Blot and Cha Raan!!!)
27. Thaw GEL tubes
28. Load the gel as follow: Ladder, Input, IgG, , Ladder, IP, S3, Ladder. (you can use 15µl for several runs)
29. Continue with the Western blot.
30. After blotting and between washes and blocking steps, cut the blot at the expected sizes for the proteins to test. LABEL the MW of the fragments!
31. Continue with the western blot, put your antibodies, washes, incubation and washes. Develop the western and let the magic do its work!

THE END!
Co-Immunoprecipitation (Co-IP) protocol for *Nematostella vectensis* v2.0

(If v1.0 will not work)

1. Rinse the beads 3X (1mL) with PBS.
2. Rinse the beads 2X (1mL) with **100mM Sodium Citrate, pH 5.0**.
3. Add antibody (Ab-b) and IgG (IgG-b) to the beads (in HB 1mL), separately.
   Incubate **2 hours** RT with rotation.
4. Rinse the beads 2X (1mL) **100mM Sodium Citrate, pH 5.0**.
5. Rinse 1X (1mL) with **200mM Triethanolamine, pH 8.2**.
6. Prepare **DMP solution**: 1mL of **20mM DMP in 200mM Triethanolamine, pH 8.2 (FRESH)**.
7. Add DMP solution to Ab-b and IgG-b. Incubate **45 minutes** at RT (Crosslinking).
8. Rinse with 1X **50mM Tris pH 7.5**.
9. Add 1mL of **50mM Tris, pH 7.5**. Incubate **45 minutes** at RT (STOP Crosslinking).
10. Rinse 3X with **PBS + 0.01% Tween-20**.
11. Block Ab-b and IgG-b with 1mL of BSA or NGS (200µg/mL) in HB.
12. Keep Ab-b and IgG-b at **4°C** until use.
13. Add IgG-b to S1 and incubate for **1 hour** at **4°C**.
14. Capture beads. Transfer supernatant (S2) to a new tube. Add WB to MB1. **DO NOT LET THEM DRY!!!** Wash 3x5 minutes at **4°C** with rotation.
15. Incubate S2 with Ab-b (Ab1) at **4°C** with rotation **O/N**.
16. Elute MB1: add **15µl of 2xAB** and pipette up/down, add **12.6µl of MiliQ water, BromoPhenol Blue (BPB)** and Beta-MercaptoEthanol (BME) to final concentrations of **0.02%** (1.2µl of 0.5% BPB for every 30µl sample) and **4%** (1.2µl of BME for every 30µl of sample). Elute pipetting up & down several times (E-MB1).
17. Incubate E-MB1 at **80°C** for **10 minutes** (in thermocycler).
18. Centrifuge E-MB1 at **max speed** for **5 min** and collect supernatant (IgG GEL).
   Snap freeze and keep it until you load the gel.

**Day II**

32. Capture beads. Transfer supernatant (S3) to a new tube. Add WB to IP. **DO NOT LET THEM DRY!!!** Wash 3x5 minutes at **4°C** with rotation.
33. Take **15-20µl of S3** and add **15µl of 2xAB**, BromoPhenol Blue (BPB) and Beta-MercaptoEthanol (BME) to final concentrations of **0.02%** (1.2µl of 0.5% BPB for every 30µl sample) and **4%** (1.2µl of BME for every 30µl of sample) (S3 GEL).
   Snap freeze the rest of S3.
34. Incubate S3 GEL at **80°C** for **10 minutes** (in thermocycler). Snap freeze and keep it until you load the gel.
35. Elute IP: add **15µl of 2xAB** and pipette up/down, add **12.6µl of MiliQ water**, add BromoPhenol Blue (BPB) and Beta-MercaptoEthanol (BME) to final
concentrations of 0.02% (1.2µl of 0.5% BPB for every 30µl sample) and 4% (1.2µl of BME for every 30µl of sample). Elute pipetting up & down several times (E-IP).

36. Incubate E-IP at 80°C for 10 minutes (in thermocycler).
37. Centrifuge E-IP at max speed for 5 min and collect supernatant (IP GEL). Snap freeze and keep it until you load the gel.