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 Na_3VO_4
- 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

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

1. 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**.
2. 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 **4ºC** 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 Raaan!!!)

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 **4ºC** until use.
13. Add **IgG-b** to **S1** and incubate for **1 hour** at **4ºC**.
14. 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.
15. Incubate **S2** with **Ab-b** (**Ab1**) at **4ºC** 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 **80ºC** 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 **4ºC** 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 **80ºC** 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 **80ºC** 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.