

## Crosslinking and Immunoprecipitation in Zebrafish

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**[Abstract]** Immunoprecipitation (IP) is a routine method to detect protein binding and interactions. But the weak binding between two proteins is often hard to detect during a regular IP procedure. This protocol offers a crosslinking and IP combination method to detect weak binding of proteins in zebrafish embryos.

### **Materials and Reagents**

1. NaCl
2. KCl
3. CaCl<sub>2</sub>
4. Tris
5. EDTA
6. Glycerol
7. Triton X-100
8. TBST
9. Glycine
10. NP-40
11. Na deoxycholate
12. SDS
13. DTSSP (Thermo Fisher Scientific, catalog number: 21578)
14. HEPES (Life Technologies, Invitrogen™, catalog number: 15630)
15. Protease inhibitors (Sigma-Aldrich, catalog number: P2714)
16. Anti-HA affinity matrix (Roche Diagnostics, catalog number: 11815016001)
17. Anti-FLAG M2 affinity gel (Sigma-Aldrich, catalog number: A2220)
18. Modified Ringer's solution (see Recipes)
19. Lysis buffer (see Recipes)
20. RIPA (see Recipes)
21. Wash buffer (see Recipes)

## **Equipment**

1. Kontes tubes

## **Procedure**

1. Protein analysis
  - a. Inject embryos with RNA encoding epitope-tagged factors at the one cell stage, manually dechorionate, and allow to develop until early gastrulation (shield stage, 6 hpf).
  - b. Determine amount of RNA to be injected by the amount needed to rescue respective mutants or morphants.

*Note: The RNA amount determines the number of embryos processed for each IP, with more embryos required in cases where relatively less RNA was injected per embryo.*
2. Crosslinking of receptors
  - c. Place embryos at shield stage in 0.2 ml modified Ringer's solution containing 5 mM DTSSP.
  - d. Incubate embryos at 28 °C for 1.5 h, then transfer into Ringer's plus 50 mM Tris pH 7.6 and incubate at room temperature for 20 min to quench the crosslinking reaction.

*Note: No crosslinking was performed for ligand IPs.*
  - e. Transfer embryos into 0.2 to 0.4 ml lysis buffer, disrupt manually in Kontes tubes with pestle, and incubate on ice for 30 min with vortexing every 5 min.
  - f. Clarify by 30 min centrifugation, and transfer supernatant to fresh tubes.
3. Immunoprecipitation (IP)
  - g. For HA epitope, use 2 µl packed resin per sample anti-HA affinity matrix added directly to embryo lysates.
  - h. For FLAG epitope, use 2.5 µl packed gel per sample anti-FLAG M2 affinity gel prepared by washing four times briefly in excess TBST, once for 10 min in 0.1 M glycine pH 3.5, four times briefly in TBST, and once in lysis buffer.
  - i. Expose samples to affinity gel overnight at 4 °C with gentle mixing.
  - j. Wash receptor IPs six times in RIPA for one hour per wash, followed by one overnight wash.
  - k. Wash ligand IPs three times briefly in wash buffer.
  - l. After washes, leave affinity resin in 10 µl buffer, then add 10 µl 2x SDS loading buffer.
  - m. Store samples at 4 °C until SDS-PAGE analysis.

## Recipes

1. Modified Ringer's solution (pH 7.8)

116 mM NaCl

3 mM KCl

4 mM CaCl<sub>2</sub>

5 mM HEPES

2. Lysis buffer

50 mM Tris (pH 7.5)

150 mM NaCl

1 mM EDTA

10% glycerol

1% Triton X-100

Protease inhibitors

3. RIPA

50 mM Tris (pH 8.0)

150 mM NaCl

1% NP-40

0.5% deoxycholate

0.1% SDS

Protease inhibitors

4. Wash buffer

50 mM Tris (pH 7.6)

150 mM NaCl

1% Triton X-100

Protease inhibitors

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This protocol was adapted from Reference 1, and tested and developed in the Michael Granato Lab at University of Pennsylvania, Philadelphia, USA. This work was supported by NIH grant R01HD037975.

## References

1. Little, S. C. and Mullins, M. C. (2009). [Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis](#). *Nat Cell Biol* 11(5): 637-643.