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 crossliking and IP combination method to detect weak binding of proteins in zebrafish embryos.

Materials and Reagents

1. NaCl
2. KCl
3. $CaCl_2$
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 $CaCl_2$
   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

Acknowledgments

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.

