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Co-Immunoprecipitation on *Drosophila* Cells in Culture

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Summary

Coimmunoprecipitation (coIP) provides evidence that two or more proteins can be found in the same complex. It can be performed in vitro (employing in vitro transcribed and translated proteins, or proteins expressed in *Escherichia coli*) or from transfected cells, which assess whether the interaction takes place in a more functional context. This chapter includes a general description and guidelines to carry out coIP in transfected Schneider's cells.

Key Words: Coimmunoprecipitation; coIP; protein G-Sepharose; S2 cells.

1. Introduction

It may prove difficult to assess the nature of certain protein–protein interactions in intact organisms. The complexity brought about by a restricted spatial distribution, the abundance of the proteins of interest, and particularly the quality and availability of specific antibodies may hinder detection. On the other hand, heterologous systems such as yeast two-hybrid assays, powerful and simple to perform as they are, may not always reflect the in vivo scenario. In that regard, immunoprecipitation from transiently or stably transfected cell lines offers distinct advantages: proteins of interest may be tagged (no protein-specific antibodies are required) and expressed at different levels from native, constitutive, or inducible promoters. Native promoters will ensure a condition closest to in vivo. However, this approach involves much more construct-building, and expression will ultimately depend on the presence of specific transcription factors in the selected cell line. Co-immunoprecipitation (coIP) from cells in culture offer another advantage: the identification of the interaction domains by deletion mapping or critical point mutations; a number of constructs can be tested at once without the requirement of generating transgenic organisms for each particular one.

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Immunoprecipitations from transfected Schneider 2 (S2) cells have been employed to confirm interactions between CRYPTOCHROME (CRY) and TIMELESS (TIM) ***(1,2)*** and CRY and PERIOD (PER) ***(3)***. These groups reported coIP to work employing a stable S2 line expressing either *tim* ***(1)*** or *per* ***(3)*** under the *Drosophila actin5C* promoter, which was then transfected transiently together with a tagged version of *cry* (either CRY-GFP or HA-CRY). Noteworthy, certain interactions depend on the proper environmental condition, such as absence or presence of light; it is important to take this matter into account when trying to reproduce the in vivo scenario.

AU: Please define GFP, if appropriate.

2. Materials

1. Schneider's S2 cells.
2. Serum-containing medium (SCM): Schneider's cells medium, 10% fetal calf serum heat-inactivated at 56°C for 30 min, 50 U penicillin G, 50 µg/mL streptomycin sulfate, filter-sterilized.
3. Sterile pipets and technique.
4. Laminar flow hood.
5. Quiet drawer or incubator: 22 to 25°C.
6. Purified plasmid DNA resuspended in TE buffer.
7. Lipid-based reagents: Effectene (Qiagen).
8. Tissue-culture treated Corning (Costar) 6- and 12-well culture clusters.
9. 10X Phosphate-buffered saline (PBS) buffer: 11.5 g/L Na_2HPO_4, 2 g/L KH_2PO_4, 80 g/L NaCl, 2 g/L KCl, pH 7.4. Dilute to 1X PBS before use.
10. ES2 protein extraction buffer: 20 m*M* HEPES, pH 7.5, 100 m*M* KCl, 0.05% Triton X-100, 2.5 m*M* EDTA, 5 m*M* dithiothreitol, 5% glycerol, 10 mg/mL aprotinin, 10 mg/mL leupeptin, and 2 mg/mL pepstatin ***(4)***.
11. Protein G Sepharose (Gammabind Plus Sepharose, Pharmacia Biotech) (*see* **Note 1**).
12. Rotisserie shaker.
13. Bicinchoninic acid (BCA) protein assay reagent kit (Pierce cat. no. 23227).
14. ELISA plate reader (or spectrophotometer).
15. TBS: 35 m*M* Tris-HCl, pH 7.4, 140 m*M* NaCl.
16. 0.05 *M* Tris-HCl, pH 6.8.
17. 10X Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel running buffer: 30.3 g/L Tris base, 144 g/L glycine, 100 mL/L SDS 10%). Dilute to 1X before use.
18. Tris-glycine transfer buffer: 3.03 g/L Tris base, 14.4 g/L glycine, 150 mL/L methanol; pH 8.4 (*see* **Note 2**).
19. 2X Laemmli sample buffer: 62.5 m*M* Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 710 m*M* β-mercaptoethanol.

ED: x-ref to Chapter 33 ok?

3. Methods

A number of transfection protocols are provided in Chapter 33. An alternative for transient transfections of Schneider S2 cells will be included here.

3.1. Transient Transfection of Suspension Cells With Effectene (see Note 3)

1. Split the cells 1:2 the day before transfection.
2. On the day of transfection, remove the cells by pipetting up and down, harvest by centrifugation (100*g*), remove the medium, and wash once with 1X PBS (*see* **Note 4**).
3. Seed the cells at a density of 1.6×10^6 cells per well in a 6-well microtiter plate in 0.8 mL SCM (*see* **Note 5**).
4. Dilute 1.6 µg of DNA dissolved in TE (*see* **Notes 6** and **7**), with the DNA-condensation buffer EC (provided in the Effectene kit), to a total volume of 100 µL. Add 12.8 µL of enhancer (provided in the Effectene kit) and mix by vortexing for 1 s (*see* **Note 8**).
5. Let it sit for 5 min at room temperature (15–25°C).
6. Add 30 µL of Effectene to each DNA-enhancer mix and pipet up and down five times, allowing 5 to 10 min at room temperature to allow transfection complex to form.
7. Dilute the complexes up to 0.8 mL in 10% SCM and add to each well dropwise (*see* **Note 9**). Gently swirl the dish to ensure uniform distribution of the complexes.
8. Cover with Parafilm. Place in an incubator at room temperature (22–25°C).
9. Determine the optimal harvesting time (*see* **Note 10**). Transfection usually proceeds for 24 to 48 h (*see* **Note 11**).

3.2. Protein Extraction and Coimmunoprecipitation

1. Resuspend the transfected cells by gently pipetting up and down, and transfer to a 1.5-mL microcentrifuge tube. If too many cells were left in the well, add 1X PBS and repeat the operation.
2. Spin down at 100*g* for 2 min.
3. Remove the SCM and add an equal volume of 1X PBS to wash out residual SCM. Resuspend the cells carefully, so as not to break them open.
4. Spin down at 100*g* for 2 min and remove most of the PBS.
5. Resuspend the cells in approx 100 µL/well of ice-cold ES2 ***(4)*** lysis buffer (*see* **Note 12**). Detergent-insoluble material is removed by centrifugation at maximum speed in a refrigerated microcentrifuge, and the soluble fraction is transferred to a new 1.5-mL microcentrifuge tube.
6. Set aside a 5- to 10-µL aliquot to determine protein concentration using the BCA assay (BCA protein assay kit), and 10 to 20 µL as a positive control for Western blots.
7. In the meantime, follow the manufacturer's recommendation to resuspend the protein-G Sepharose beads and generate a 1:1 (v/v) slurry.
8. Wash the beads with 1X PBS; centrifuge at 100 to 200*g* for 2 min and remove the excess PBS to return to the original 1:1 slurry.
9. Incubate the lysate with 10 µL of beads for 30 min in a rotisserie shaker in a slow head-to-tail motion (*see* **Note 13**).

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10. Spin down at 100*g* for 2 min and transfer the supernatant to a new microcentrifuge tube.
11. Add 0.5 to 5 µL of specific antibody to each lysate. Incubate on ice (or in a rotisserie shaker at 4°C) for 1 to 3 h. Include proper controls (lysates lacking the proteins of interest immunoprecipitated in parallel; a primary antibody against another protein known to be present or complexed in the lysate).
12. Add up to 50 µL of the slurry per mL lysate. Mix with gentle shaking for 1 h at 4°C.
13. Wash the antigen–antibody–protein G complexes by centrifuging for 1 min at 200*g*. Carefully aspirate the supernatant with a fine tipped Pasteur pipet. Gently resuspend in 1 mL ES2. Repeat the wash. Wash in TBS and in 0.05 *M* Tris-HCl, pH 6.8 (*see* **Note 14**).

AU: Tris-HCl OK in step 13?

3.3. Preparation for SDS-PAGE

1. Microcentrifuge at 200*g* for 1 to 2 min. Gently, without splatter, add 20 to –50 µL 1X Laemmli sample buffer. **Do not vortex.** Heat for 5 min at 100°C. Microcentrifuge as above and apply supernatant directly to an SDS-PAGE. Make sure to include positive controls (transfected cells that have not been immunoprecipitated) as well as negative controls.
2. Run a 6% SDS-PAGE to detect large proteins such as PER, TIM, or CLOCK, or a gradient gel (4–12%) when analyzing proteins of very different molecular weights.
3. Transfer to supported nitrocellulose in a Tris–glycine buffer. Perform Western blots as usual.

4. Notes

1. According to Pharmacia, the binding capacity of Gammabind Plus is relatively low to pull down hamster, rat, and mouse immunoglobulins. When employing primary antibodies from any of those species it is highly recommended to switch to an anti-species-IgG coupled to agarose beads.
2. When preparing transfer buffer measure the pH but do not adjust it. If it is not the one indicated, prepare a new solution.
3. Preliminary experiments showed a somewhat higher transfection efficiency employing Effectene, although cell integrity appeared more affected than in the presence of other lipid-based reagents. Transfection efficiency might affect to a higher extent on coIP than on transcriptional assays and may hamper the detection of the desired interaction. Keep in mind that the overall efficiency revolves around 10% of the transfected cells. In such cases it is recommended to generate a stable cell line expressing a tagged protein (to facilitate further detection) and transiently transfect the other constructs. Cells transfected in different wells can be pooled after transfection to improve detection of the desired interaction.
4. The wash may be skipped without a substantial loss in transfection efficiency.
5. Cells can be plated at 1 to 3.5×10^6 cells in 1.6 mL SCM per well in a 6-well microtiter plate.

6. DNA should be resuspended in TE, pH 7.0 to 8.0, at a minimum concentration of 0.1 μg/μL. When transfecting two constructs altogether use half of the total DNA amount of each.
7. Plasmid DNA quality strongly influences several transfection parameters such as efficiency, reproducibility, and toxicity, as well as interpretation of results. Therefore only plasmid DNA of the highest purity should be used.
8. It is important to always keep the ratio of DNA to enhancer constant.
9. It is not necessary to stress the cells by placing them in serum-free medium, as serum does not inhibit Effectene.
10. In many cases, removal of transfection complexes is not necessary. However, if cytotoxicity is observed, centrifuge cells after 6 to 18 h, remove medium containing the complexes, wash the cells with 1X PBS, then resuspend in 1.6 mL fresh growth medium.
11. When transfecting a reporter such as GFP the optimal harvesting time can be easily determined by inspecting the cells under an inverted fluorescent microscope. It will require at least 6 to 8 h posttransfection is to identify GFP-expressing cells.
12. The detection of the desired protein–protein interaction will depend greatly on the salt concentration of the medium. Higher salt concentration will select for more specific interactions, but weak interactors may be lost. When no information is available, it is recommended to perform coIP in buffers of different ionic strength ranging from 25 to 150 m*M* KCl. Add the protease inhibitors right before use.
13. This clearing step in the absence of the specific primary antibody allows the removal of the complexes formed between the protein G coupled to the beads and any protein present in the lysate.
14. Make sure to perform at least four washes.

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