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IMMUNOPRECIPITATION FOR ANALYSIS OF RADIOLABELED CELL SURFACE MOLECULES

General:

The purpose is molecular characterization of antigens identified by specific antibodies. Both conventional and monoclonal antibodies may be employed. Monoclonals are usually non-precipitating, as are most conventional antibodies at the minute antigen concentrations employed. Therefore a second, indirect or 'sandwich' reagent is employed to induce precipitation, either anti-Ig, purified anti-Ig coupled to Sepharose, or Staph aureus Cowan strain II bacteria (the latter is not generally applicable to the rat, where only IgG1 and IgG2c subclasses bind). Recently, excellent results have been obtained by using mouse anti-rat or rat anti-mouse K chain MAb as second antibodies in conjunction with S. aureus bacteria. Generally, the antigens are maintained in solution by detergents, nonionics being preferable over bile salts which may precipitate due to the pH or divalent cations. Detergent should be present in at least 5 x the concentration of lipid (5 mg/ml in serum) in the antibody preparations, and should be maintained in washes until the last, where it is removed to prevent interference with SDS. Whole serum anti-Ig's are preferable to IgG fractions in terms of lower backgrounds. However, anti-Ig coupled to Sepharose should be first affinity purified. It should be remembered that in SDS and reducing agent, all Ig chains except the ~ 1/4 actually linked to Sepharose will be eluted. Distortion of the gel pattern by overloaded zones containing the cold H and L chains is a problem of the technique. This will often be noticed as an area from which label is displaced. The anti-Ig-Sepharose method is quicker from start to finish than the serum anti-Ig method, but the actual work involved is not much less. The patterns obtained with serum anti-Ig and anti-IgG Sepharose are comparably specific. Sepharose CL-4B appears to give a lower background than CL-2B, perhaps because aggregates are better excluded. Use of monoclonal anti-IgG's such as RG7/9 or RG7/7 coupled to Sepharose is highly advantageous. Coupling the anti-surface antigen MAb directly to Sepharose is even better.

1. Antigen. Labeled, detergent solubilized antigen is usually stored at -80°C and used in 1% Triton X-100, 1% bovine hemoglobin, 0.14 M NaCl, 0.01 M tris-HCl, pH 8.0 (solubilization buffer).

2. Pre-precipitation. A mock precipitation with the sandwich reagent is carried out to clear labeled surface IgM, IgD, Fc receptor-bound IgG, and non-specifically precipitating material. Usually this is done with the entire lot of an antigen batch. Serum anti-Ig: Add normal serum as a source of carrier IgG (1 µl/ml of radiolabeled antigen), add the proper equivalent of anti-Ig serum, 12-18 h 4°, centrifuge (1000 g, 10 min) or microfuge and reserve sup. Anti-Ig-Sepharose: Shake with ~ 20 µl of 10 mg/ml pure anti-Ig-Sepharose CL-4B; 1:1 slurry, per ml of antigen, 1.5 h 4°, centrifuge, reserve sup. Note: pre-precipitation is more complete if done with a homologous antibody, e.g. for mouse sIg, use anti-mouse IgG rather than anti-rat IgG. Pre-precipitation is less important with MAb-Sepharose or RG7/7-Sepharose.

3. Precipitations:

Precoat 1.5 ml Sarstedt conical tubes without caps with 1% Hb in TSA. Add in order wash buffer, 30 µl (cloned line) or 100 µl (uncloned line) of culture supernatant, and mix with 25-200 µl labeled antigen containing 10^5 - 10^6 cpm of ^{125}I (lactoperoxidase) or ^{35}S (for fluorography), bringing total volume to ideally 200 µl. Vortex and allow to stand 2 h, 4°C and cover microtiter plate.

A. Serum anti-Ig: Centrifuge the anti-Ig if aggregated material is present. Add an excess of anti-Ig over the first antibody. This can be determined by titrating the amount of labeled antigen precipitated by a constant amount of first antibody and a varying amount of second antibody. For rabbit anti-rat IgG, 11-20-78, the amount to use is 1/3 the volume of the culture supernatant containing the first antibody. Considerably more second antibody would be required for serum antibodies (~ 20-40 µl/µl). Mix and allow to stand 12-18 h at 4°.

B. Anti-Ig Sepharose: Use same volume of a 1:1 slurry of 10 mg pure anti-Ig/ml Sepharose CL-4B as anti-Ig serum above. Make to volume of 0.2-0.5 ml with wash buffer and shake at '7' on microshaker 1.5 h 4°.

C. MAb-Sepharose: Add ~ 10 µl of 1:1 slurry of ~2 mg MAb/ml Sepharose CL-4B and shake as in 'B' in a total volume of 200 µl.

4. Washing:

Wash buffer: 0.1% Hb, 0.1% Triton X-100, 0.14 M NaCl, 0.01 M Tris-HCL pH 8.0. Centrifugation for immunoprecipitates is at top speed (1,000 x g) x 7 min. For Sepharose, is 200 x g x 2 min.

First wash: Dilute with wash buffer to ~1.4 ml and centrifuge. Aspirate with a pasteur-pipette with constricted tip, leaving ~10 µl fluid above pellet.

Second wash: 1.4 ml wash buffer.

Third wash: Tris-saline, pH 8.0.

Fourth wash: 0.05 M Tris-HCl, pH 6.8 (similar to sample buffer). Aspirate most of the supernatant. Centrifuge to bring down any residual drops on side of tubes and aspirate again, leaving ~ 10 µl over pellets.

5. For SDS gel electrophoresis on 1.6 mm thick gels:

Add 40 µl of SDS sample buffers containing 3% SDS. Do not vortex as pellets tend to stick to sides of tubes. Cap and secure caps by placing a lid over them and taping it down. For immunoprecipitates, incubate at 56° x 1 h. The 56° incubation should completely dissolve the pellets, avoiding irreversible aggregation which occurs when insoluble proteins not accessible to SDS are immediately boiled. Next, place the tubes in a tupperware

container with freshly-boiled water for 10 min. Vortex, and centrifuge 1 min at 200 x g. Cool, and samples are now ready to be applied to SDS gels (see 'SDS slab gel electrophoresis').

Controls:

positive control supernatant (e.g., M1/9.3.4)
negative control (e.g., NSI sup. + 50 µg/ml normal rat IgG).