User Manual

Antibodies Protocol Guide

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Table of Contents

I. Introduction

Clontech's Antibodies Protocol Guide provides experimental procedures for commonly used antibody techniques. These protocols are intended as starting points for establishing optimal conditions for your experimental system. In some instances, specific, optimized antibody protocols are required. When this is the case, we supply the preferred protocols with the antibody. These optimized protocols should be used instead of the more generalized procedures in this guide.

Please note that not all of our antibodies have been tested using each of the techniques presented here. Consult the Product Analysis Certificate (PAC) included with your antibody for a list of confirmed techniques. Additionally, please refer to the PAC for antibody-specific and lot-specific information such as recommended dilutions. The Antibodies Protocol Guide includes procedures for Western blotting, immunoprecipitation, immunofluorescence, immunocytochemistry, and ELISA. An additional list of references for antibody techniques is provided in Section VII.

II. Materials Required

A list of required materials for each technique is provided at the beginning of each section.

All protocols are written using phosphate buffered saline (PBS). For most of the techniques, Tris-based buffers may be substituted. Prepare 5X PBS as follows:

5X Phosphate-buffered saline (PBS; pH 7.4)

		5X conc.	1X conc.
82.3 g	Na_2HPO_4	0.29 M	58 mM
20.4 g	NaH_2PO_4	0.085 M	17 mM
40.0g	NaCl	0.34 M	68 mM

Adjust to pH 7.4 with 0.1 N NaOH; add distilled H_2O to a final volume of 2 L. Store at room temperature. Dilute to 1X before use.

III. Western Blotting

Materials Required

- **1X PBS** (Alternatively, you may substitute Tris-based buffers.)
- **2X SDS sample buffer** (see Sambrook et al., 1989)
- **Wash buffer** (0.2% Tween-20 in PBS, or TBS)
- **Blocking buffer** (5% nonfat dry milk* in wash buffer)

  * For phosphotyrosine antibodies, substitute with 3% BSA.

  It may be necessary to heat the solution slightly to fully dissolve nonfat dry milk. Store at 4°C (stable for up to 1 week).
- **Secondary antibody**
  Use either an alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated secondary antibody directed against the host species of your primary antibody.
- **AP or HRP detection system** (chemiluminescent or colorimetric)

A. Preparation of Mammalian Cell Lysate and Electrophoresis

Note: For preparation of yeast protein extracts, please refer to Clontech's Yeast Protocols Handbook (PT3024-1; available at **www.clontech.com**).

1. Collect the cells from one 100-mm dish (or 2–5 x 10^7 cells for suspension cultures) by centrifuging at 2000 rpm for 10 min at room temperature.
2. Wash the cell pellet twice with 0.5 ml PBS; spin and remove supernatant after each wash.
3. Add 0.3 ml PBS and disrupt cells by sonication. Alternatively, disrupt cells by repeatedly passing suspension through a 21-gauge needle.
4. For **total cell lysates** (which include the nuclear fraction), proceed to the following step. For **soluble fraction lysates** spin the crude lysate at 14,000 rpm for 30 min at 4°C. Collect the supernatant.
5. Measure the protein concentration using standard techniques.
6. Mix 20 µl (about 40 µg) lysate with an equal volume of 2X SDS sample buffer. Boil 3 min and spin down.
7. Load 10 µl boiled lysate on a polyacrylamide gel and perform SDS electrophoresis using standard procedures.

III. Western Blotting continued

B. Western Blotting

Note: Optimal dilutions and incubation times may vary with individual systems and must be determined empirically.

1. Transfer proteins from the gel to a nitrocellulose or PVDF membrane using standard techniques.
2. Add 20 ml blocking buffer and incubate membrane for 1 hr at room temperature with shaking. Alternatively, incubate at 4°C overnight.
3. Dilute the primary antibody in blocking buffer according to the specifications on the Product Analysis Certificate (or dilute to a concentration of ~1 µg/ml). **If you are using a Clontech primary antibody directly conjugated to HRP or AP, skip to Step 6.**
4. Incubate the membrane with the diluted antibody for 2 hr at room temperature with shaking.
5. Wash the membrane two times with wash buffer for 5 min each wash.
6. Dilute a secondary antibody conjugate 1:1,000–20,000 in blocking buffer. If you are using a conjugated primary antibody refer to the suggested dilution on the Product Analysis Certificate.
7. Incubate the membrane with the diluted antibody for 1 hr at room temperature with shaking.
8. Wash the membrane four times with wash buffer for 10 min each wash.
9. Proceed with an appropriate chemiluminescent or colorimetric detection method.

IV. Immunoprecipitation

Materials Required

- **1X PBS**
- **Lysis buffer** (stock solution)
  Prepare a lysis buffer stock solution without protease inhibitors as follows:

		Final conc.
100 ml	5X PBS	1 X
5 ml	0.5 M EDTA	5 mM
2.5 ml	Triton X-100*	0.5 %

  * Alternatively you may substitute NP-40 (0.5% final concentration)

  Add distilled H_2O to 500 ml final volume. Store at 4°C.
- **Lysis buffer** (working solution)
  At the time of your experiment, add the following protease inhibitors to a sufficient aliquot of stock lysis buffer. You will need 3 ml per 100-mm dish.

  Note: Alternatively, you may use inhibitors that are optimized for you experimental system.

	Final conc.
PMSF	0.1 mM
pepstatin A	10 µM
leupeptin	10 µM
aprotinin	25 µg/ml

- **Immunopurification reagent**
  We recommend our Protein LA-Agarose (#3904-1, -2) for high affinity purification of a variety of immunoglobulins. You may also use Protein A or Protein G agarose as appropriate for your antibody.
- **2X SDS sample buffer** (see Sambrook et al., 1989)

A. Preparation of Mammalian Cell Lysate

Note: For preparation of yeast protein extracts, please refer to Clontech's Yeast Protocols Handbook (PT3024-1; available at **www.clontech.com**).

1. Collect cells from one 100-mm dish (or 2–5 x 10^7 cells for suspension cultures) by centrifuging at 2000 rpm for 10 min at room temperature.
2. Wash the cell pellet twice with 0.5 ml PBS; spin and remove supernatant after each wash.
3. Add 3 ml lysis buffer. Incubate for 30 min at 4°C on a rotating apparatus.
   Alternatively, you may disrupt cells by repeatedly passing the sample through a 21-gauge needle.
4. Clear the lysate by centrifuging at 12,000 rpm for 30 min at 4°C. Collect supernatant.

IV. Immunoprecipitation continued

B. Immunoprecipitation

1. Transfer 1.2 ml cleared lysate to a microcentrifuge tube. Add 20 µl protein LA/A/G-agarose beads.
2. Incubate for 1 hr at 4°C on a rotating apparatus to remove non-specifically bound proteins.
3. Spin down beads and transfer supernatant to a new microcentrifuge tube.
4. Add 2–5 µg antibody to supernatant. (Optimal amount of antibody may vary.)
5. Add 25 µl protein LA/A/G-agarose beads.
6. Incubate for 4–6 hr or overnight at 4°C on a rotating apparatus.
7. Spin down beads; remove supernatant.
8. Wash beads five times with 1 ml PBS for 2 min each wash.
9. Discard supernatant from final wash and resuspend pellet in 25 µl 2X SDS sample buffer. Boil samples for 5 min and spin down.
10. Load 10–15 µl of supernatant on an SDS/polyacrylamide gel. Continue with Western blotting as described in Section III.B.

V. Immunofluorescence and Immunocytochemistry cont.

Materials Required (immunofluorescence **and** immunocytochemistry)

- **1X PBS**
- **Fixative** (see Step A.3, below)
- **PBS-BSA** (2% BSA in PBS; prepare fresh)

Additional materials required (immunofluorescence only)

- **2% goat serum in PBS-BSA**
- **Secondary antibody**
  Use FITC- or Texas Red-labeled goat anti-mouse or anti-rabbit antibody as appropriate. Other labels can also be used.
- **Mounting medium**
  Use one of the following:

  –aqueous mounting medium
  –90% glycerol in PBS
  –anti-fade medium

Additional materials required (immunocytochemistry only)

- **10% goat serum in PBS-BSA**
- **0.5% Triton X-100 in PBS**
- **DAB solution**
- **Secondary antibody**
  Use a biotin-conjugated secondary antibody and streptavidin- or avidin-conjugated horseradish peroxidase (HRP).

A. Sample Preparation

1. Grow cultured cells on sterile cover slips or slides overnight at 37°C.
2. Remove medium and rinse briefly with 1X PBS
3. Fix cells using **one** of the following procedures:
   - 1% formalin in PBS; treat 10 min and keep wet.
   - Methanol (–20°C); treat 3 min and air dry.
   - Acetone (–20°C); treat 2 min and air dry.
4. Rinse the cover slips or slides three times with PBS.
5. Continue with the procedure for immunofluorescence (Section B) or immunocytochemistry (Section C).

V. Immunofluorescence and Immunocytochemistry cont.

B. Immunofluorescence

Carry out all incubations at room temperature in a humidified chamber with gentle shaking. Use suction to remove reagents after each step. Do not allow samples to dry out.

1. Block samples by incubating cover slips with PBS-BSA. Use sufficient volume to cover the glass (about 100 µl). Incubate for 20 min.
2. Wash once with PBS.
3. Dilute primary antibody to 2–5 µg/ml in PBS-BSA. Optimal dilution should be determined empirically (typically 1:200–500).
4. Incubate with primary antibody for 30 min.
5. Wash three times with PBS for 5 min each wash.
6. Incubate with 2% goat serum in PBS-BSA for 20 min.
7. Wash two times with PBS for 5 min each wash.
8. Dilute secondary antibody according the manufacturer's directions in PBS-BSA.
9. Incubate with secondary antibody for 30 min in the dark.
10. Wash five times with PBS for 5 min each wash.
11. Mount coverslips with mounting medium.
12. Examine using a fluorescence microscope with appropriate filters.

C. Immunocytochemistry

Carry out all incubations at room temperature in a humidified chamber with gentle shaking. Use suction to remove reagents after each step. Do not allow samples to dry out.

1. Quench endogenous peroxidase activity by incubating cover slips in 0.5% peroxide for 10 min.
2. Wash two times with PBS for 5 min each wash.
3. Block samples by incubating cover slips with 10% goat serum in PBS-BSA. Use sufficient volume to cover the glass (about 100 µl). Incubate for 30 min.
4. Wash two times with PBS for 5 min each wash.
5. Dilute primary antibody to 2–10 µg/ml in PBS-BSA. Optimal dilution should be determined empirically.
6. Incubate with primary antibody for 60 min at room temperature (or overnight at 4°C).
7. Wash three times with PBS for 5 min each wash.
8. Dilute biotin-conjugated secondary antibody according to the manufacturer's directions in PBS-BSA (typically 2–10 µg/ml).
9. Incubate with secondary antibody for 45 min at room temperature.

V. Immunofluorescence and Immunocytochemistry cont.

10. Wash three times with PBS for 5 min each wash.
11. Dilute streptavidin- or avidin-conjugated HRP according to the manufacturer's directions in PBS-BSA (typically 1:100–500).
12. Incubate with HRP-conjugate for 20 min.
13. Wash thoroughly (more than five times) with PBS for 5 min each wash.
14. Rinse in 0.5% Triton X-100 in PBS for 30 sec.
15. Incubate in DAB solution for 5 min.
16. Monitor development of color under a microscope. If further intensification of staining is required, return to DAB and incubate for an additional 1–5 min.
17. Rinse slides in distilled H_2O.
18. Counterstain in hematoxylin, if desired.
19. Dehydrate through alcohols and xylene.
20. Permanently mount coverslip and observe with light microscopy.

VI. ELISA

Materials Required

- **1X PBS**
- **Coating buffer** (100 mM carbonate buffer, pH 9.6)
- **Blocking buffer** (1% BSA in PBS)
- **Wash buffer** (0.05% Tween-20 in PBS)
- **Secondary antibody**
  Use either an alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated secondary antibody directed against the host species of your primary antibody. You will also need the appropriate detection substrate.

Procedure

1. Coat a microtiter plate with target antigen. Use 0.5–1 µg of antigen in 100 µl coating buffer per well. Optimal concentration should be determined empirically. Alternatively, coat wells with cell lysate supernatant.
2. Cover plate with parafilm and incubate overnight at 4°C . Alternatively, incubate at room temperature for 8 hr or at 37°C for 2 hr.
3. Invert plate and shake dry to remove antigen solution.
4. Add 200 µl blocking buffer per well. Incubate 2 hr at room temperature or overnight at 4°C.
5. Remove blocking buffer; wash plates two times for 3 min with 200 µl wash buffer per well.
6. Dilute antibody samples and controls in blocking buffer, and add 100 µl per well. Incubate 1 hr at room temperature with shaking.
7. Wash plates three times for 3 min with 200 µl wash buffer per well.
8. Dilute secondary antibody according to the manufacturer's directions in blocking buffer (typically 1:500–5,000).
9. Add 100 µl diluted secondary antibody per well, and incubate 1 hr at room temperature with shaking.
10. Wash plate five times for 3 min with 200 µl wash buffer per well.
11. Add 100 µl substrate solution per well; incubate from 10 min to 1 hr. Optimal incubation time should be determined empirically.
12. Read the microtiter plate.

VII. References

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