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Immunoprecipitation Kit (Protein A)

Cat. No. 11 719 394 001 20 reactions

Immunoprecipitation Kit (Protein G)

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Version 07
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Store at 2-8°C

For the immunoprecipitation of proteins from cellular extracts with protein A/G-agarose. The kits contain all reagents necessary for cell lysis, solubilization, stabilization and immunopurification of proteins.

1. Introduction

Immunoprecipitation is a widely used method for the analysis of target antigens in complex mixtures of proteins. The protein of interest can be concentrated and immunoaffinity-purified in one step on an analytical scale via a specific antibody. Often immunoprecipitated proteins are functionally fully active and can be further analyzed with respect to enzymatic activity, interactions, modifications and structure.

2. Kit features

2.1 Kit contents

1. Core buffer, 50 ml
   Solution of 250 mM Tris-HCl, pH 7.5, stabilized
2. NaCl, 50 ml
   Solution of 1 M NaCl, stabilized
3. Detergent mix, 15 ml
   Solution of 10 mM Tris-HCl, 10% Nonidet P40 and 5% sodiumdeoxycholate, pH 7.5
4. Complete
   Protease inhibitor cocktail tablets
   5 tablets
5. Protein A-agarose, 1 ml bed volume,
   (= 2 ml suspension volume),
   Suspension, ready-to-use
   or
6. Protein-G-agarose, 1 ml bed volume,
   (= 2 ml suspension volume),
   Suspension, ready-to-use

2.2 Stability and storage

Store the kit at 2-8°C, do not freeze. All kit components are stable until the expiry date indicated (see lot-specific label imprint).

3. Standard protocol

3.1 General remarks

Cell lysis and solubilization of proteins are crucial steps in immunoprecipitation. Any method should ensure solubilization of the target protein in a form that is immunoreactive, undegraded and ideally biologically active. Factors that influence the efficiency of solubilization and subsequent immunoprecipitation of proteins are the ionic strength and pH of the lysis buffer, type and concentrations of the detergents used, presence of divalent cations and other factors (1, 2). The standard protocols as described are general guidelines and first choice protocols but can be varied according to the requirements.

3.2 Preparation of working solutions and stabilities

3.2.1 Lysis buffer/ wash buffer 1 The kit contains reagents for 125 ml of lysis buffer/ wash buffer 1. To prepare 25 ml of lysis buffer/wash buffer 1 (minimal volume), combine the kit components as indicated and add water to a final volume of 25 ml. According to the protocol, 25 ml of this buffer is sufficient for four immunoprecipitations.

The solution is stable for 24 h at 2 – 8°C. When stored aliquoted at −15 to −25°C, the solution is stable for at least 4 weeks. Mix thoroughly after thawing.

Kit component	Final concentration (25 ml)
5 ml core buffer	50 mM Tris-HCl, pH 7.5
3.75 ml NaCl	150 mM NaCl
2.5 ml detergent mix	1% Nonidet P40 0.5% sodium deoxycholate
1 Complete tablet	1 tablet/25–50 ml

3.2.2 Wash buffer 2 (high salt) The kit contains reagents for 50 ml of wash buffer 2. To prepare 50 ml, combine the kit components as indicated and add water to the final volume. According to the protocol, 2 ml of this buffer is required for one immunoprecipitation.

The solution is stable at 2 – 8°C. For longer periods, store aliquoted at −15 to −25°C. Mix thoroughly after thawing.

Kit component	Final concentration (50 ml)
10 ml core buffer	50 mM Tris-HCl, pH 7.5
25 ml NaCl	500 mM NaCl
0.5 ml detergent mix	0.1% Nonidet P40 0.05% sodium deoxycholate

3.2.3 Wash buffer 3 (low salt) The kit contains reagents for 25 ml of wash buffer 3. To prepare 25 ml, combine the kit components as indicated and add water to the final volume. According to the protocol, 1 ml of this buffer is required for one immunoprecipitation. The solution is stable at 2 – 8°C. For longer periods, store aliquoted at −15 to −25°C. Mix thoroughly after thawing.

Kit component	Final concentration (25 ml)
1 ml core buffer	10 mM Tris-HCl, pH 7.5
0.25 ml detergent mix	0.1% Nonidet P40 0.05% sodium deoxycholate

3.3 Working procedure

3.3.1 Overview

Step (procedure described in section)	Solution required (preparation described in section)	Volume per assay	Total volume for 20 assays
Cell lysis and sample preparation (section 3.3.2)	Lysis buffer (section 3.2.1)	1–3 ml	20–60 ml
Preclearing of the sample (section 3.3.3)	Protein A/G-agarose suspension (ready-to-use)	50 µl	1 ml
Immunoprecipitation (section 3.3.4)	Protein A/G-agarose suspension (ready-to-use)	50 µl	1 ml
1. Wash, 2 times (section 3.3.4)	Wash buffer 1 (section 3.2.1)	2 ml	40 ml
2. Wash, 2 times (section 3.3.4)	Wash buffer 2 (section 3.2.2)	2 ml	40 ml
3. Wash, 1 time (section 3.3.4)	Wash buffer 3 (section 3.2.3)	1 ml	20 ml

3.3.2 Cell lysis and sample preparation

1. Wash cells/tissue at least twice with ice-cold PBS to remove any remaining serum proteins from the culture medium. For one immunoprecipitation reaction a sample volume of 1 – 3 ml is recommended. Using a micro-centrifuge, a volume of 1 ml is optimal.

- **Adherent cells** should be washed by addition of PBS to the monolayer and disposal of the supernatant. Add lysis buffer (cooled to 2 – 8°C) to the chilled, washed cell monolayers to achieve a concentration of 10^6 – 10^7 cells/ml. Scrape the cells to one side of the dish with a suitable device.
- **Cells in suspension** should be washed with PBS by centrifugation and resuspension of the pellet. Remove supernatant after the last wash. Resuspend the cell pellet in lysis buffer (cooled to 2 – 8°C) to achieve a concentration of 10^6–10^7 cells/ml and transfer to an appropriate homogenizing device.
- **Solid tissue** should be washed by addition of PBS and disposal of the supernatant. Add lysis buffer to the sample to achieve a concentration of 5 – 20 mg tissue/ml.

2. Transfer sample to a Dounce homogenizer, pre-chilled on ice or any other type of micro-homogenizer (be aware that the homogenization procedure might be critical for the functional integrity of the target antigen). Using a type B pestle, homogenize by repeated strokes (approx. 10).
3. Centrifuge homogenized suspension at 12 000 × g, 10 min, 2 – 8°C in a table-top microfuge to remove debris. Alternatively, to prepare a high speed supernatant, centrifuge at 100,000 × *g*, 45 min, 2 – 8°C.
4. Separate the supernatant and transfer to a microfuge tube (optimal volume 1 ml).

3.3.3 Preclearing with protein A/G agarose

To reduce background caused by non-specific adsorption of irrelevant cellular proteins to protein A/G-agarose, a preclearing step is recommended.

5. Add 50 µl of the homogeneous protein A/G-agarose suspension (25 µl bed volume) to the sample (1 – 3 ml) and incubate for at least 3 h (or overnight) at 2 – 8°C on a rocking platform.
6. Pellet beads by gravity sedimentation or alternatively by centrifugation at 12,000 × *g* for 20 s in a microfuge. Transfer supernatants to fresh tubes.

3.3.4 Immunoprecipitation of the target protein

7. To 1 – 3 ml of sample add an appropriate amount of the specific antibody (see section 4.1) and gently rock for 1 h at 2 – 8°C.
8. Add 50 µl of the homogeneous protein A/G-suspension to the mixture and incubate for at least 3 h (or overnight) at 2 – 8°C on a rocking platform.
9. Collect complexes by gravity sedimentation or alternatively by centrifugation at 12,000 × *g* for 20 s in a microfuge.
10. Remove supernatant carefully, add 1 ml of wash buffer 1, resuspend the beads and incubate for 20 min at 2 – 8°C on a rocking platform.
11. Repeat steps 9 and 10.
12. Collect complexes as described in step 9, add 1 ml of wash buffer 2 to the pellet, resuspend, incubate for 20 min at 2 – 8°C on a rocking platform, pellet the beads again and remove super-natant.
13. Repeat step 12.
14. Add 1 ml of wash buffer 3 to the pellet, resuspend, incubate for 20 min at 2 – 8°C on a rocking platform, pellet the beads again and remove supernatant.
15. Remove the last traces of the final wash from the agarose pellet and from the walls and lid of the microfuge tube.

3.3.5 Gel electrophoresis

The immunoprecipitated proteins can be separated by any type of one- or two-dimensional electrophoresis system providing sufficient protein resolution (3, 4, 5). For a detailed protocol for SDS-polyacrylamide gel electrophoresis or two-dimensional electrophoresis, please refer to one of the standard textbooks or to manuals from manufacturers of electrophoresis equipment.

- Add 25 – 75 µl of gel-loading buffer to the agarose pellet (step 15).
- Denature proteins by heating to 100°C for 3 min. Remove protein A/G-agarose by centrifugation at 12,000 × *g* for 20 s at 15 – 25°C in a microfuge. Transfer supernatant to a fresh tube.
- Analyze an aliquot by SDS-polyacrylamide gel electrophoresis.

3.3.6 Western blotting

After electrophoresis, blot the gel onto a nitrocellulose or PVDF membrane* using a standard Western blot protocol (6, 7). To avoid damage or contamination of the membrane, always wear gloves when handling.

- Hydrophobic membranes such as PVDF must be pre-wetted prior to protein transfer: Moisten the membrane with methanol for a few seconds, then soak with transfer buffer for at least 5 min. Nitrocellulose should be briefly soaked in water and then for at least 5 min in transfer buffer.
- It is essential to thoroughly equilibrate the gel in transfer buffer for 5 – 10 min prior to transfer.
- Blot according to standard protocols.
- The blot can be stored dry for several months in a refrigerator if necessary, but must be re-wetted before starting immunodetection. PVDF membranes should be re-wetted in methanol or in 5% Tween 20 (v/v) solution.

4. Additional information

4.1 Antibody concentration

1 ml of protein A/G-agarose binds about 20 mg of pure IgG, equivalent to about 2 ml of serum, 200 ml of supernatant from cultured hybridoma cells or 1 ml ascites fluid. Any individual antibody should be titrated in pilot experiments in which increasing quantities of antibody are used to precipitate a fixed amount of antigen. Usually, between 0.5 µl and 5 µl of polyclonal antiserum, 5 µl and 100 µl of hybridoma tissue culture medium, 0.1 and 1.0 µl of ascitic fluid or 1 µg and 5 µg of purified monoclonal or polyclonal antibodies are sufficient for complete immunoprecipitation.

4.2 Binding characteristics of protein A-agarose and protein G-agarose

Protein A and protein G are cell wall proteins, isolated from specific bacterial strains, and have specific binding sites for certain classes of immunoglobulins (table 1) from different species (table 2). Protein A binds (to varying degrees) IgM, IgA, IgD and most subclasses of IgG. Protein G binds nearly all subclasses of IgG, but no other classes of immunoglobulins.

Table 1: Affinities of protein A/G for various IgG subclasses.

Antibody	Protein A	Protein G
Human IgG_1	++++	++++
Human IgG_2	++++	++++
Human IgG_3	−	++++
Human IgG_4	++++	++++
Rat IgG_1	−	+
Rat IgG_{2a}	−	++++
Rat IgG_{2b}	−	++
Rat IgG_{2c}	+	++
Mouse IgG_1	+	++++
Mouse IgG_{2a}	++++	++++
Mouse IgG_{2b}	+++	+++
Mouse IgG_3	++	+++

Table 2: Affinities of protein A/G for antibodies of various species

Antibody	Protein A	Protein G
Human	++++	++++
Horse	++	++++
Cow	++	++++
Pig	+++	+++
Sheep	+/−	++
Goat	−	++
Rabbit	++++	+++
Chicken	−	+
Hamster	+	++
Guinea pig	++++	++
Rat	+/−	++
Mouse	++	++

4.3 Inhibitors

In extracts from animal tissues mainly serine, cysteine and metalloproteases are found; in plant extracts serine and cysteine proteases are dominating. Serine and metalloproteases are typical for bacterial extracts (8). Complete tablets inhibit efficiently serine, cysteine and metalloproteases in a broad range.

In rare cases aspartic proteases ("acid proteases") can interfere upon isolations in animal tissues. These proteases however exhibit pronounced activities only in the acid pH range. If extraction have to be performed at these pH values or single isolation steps are proceeded at low pH range possibly occuring aspartic protease activity is recommended to be inhibited by the addition of pepstatin*.

Complete contains EDTA in the typical working concentration. Therefore, the extraction buffer should not contain divalent cations like Ca^{2+}, Mg^{2+} or Mn^{2+}; otherwise the inhibition of the metalloproteases might be incomplete.

The protease inhibitors in the Complete tablets are not forming irreversible complexes with SH groups in proteins.

However, when working with biological material containing considerable amounts of "untypical" proteases, which are not well covered by the protease inhibitor cocktail, we recommend adding specific inhibitors if available. To protect secondary modifications (*e.g.*, phosphorylation, glycosylation) from degradation, specific inhibitors should be added to the buffers. Keeping the temperature during the whole procedure between 0°C and +4°C will help to reduce enzymatic degradation.

Table 3: Specificity of protease inhibitors

Inhibitor	Specificity	Working concentration
Complete	serine-, cysteine-,metalloproteases, calpains	1 tablet/25 – 50 ml
Aprotinin	serine proteases	0.06 – 2 µg/ml
EDTA	metalloproteases	0.5 – 5 mM
Pefabloc[4] SC	serine proteases such as trypsin and chymotrypsin	0.4 – 4 mM
Pepstatin	aspartate proteases	1 µM
Leupeptin	serine and cystein proteases such as plasmin, trypsin, papain, cathepsin B	1 – 10 µg/ml

4.4 Detergents

Detergents are essential for breaking up the cells and keeping proteins in a soluble state (particularly membrane-associated proteins). In most cases, especially when electrophoresis is the analytical step, the detergent mix delivered with the kit will be suitable. However, for some antigens, special, more sophisticated solubilization protocols may be applied when the protein has to be obtained in a functionally active state (9, 10).

4.5 Wash conditions

Different buffers are commonly used to wash protein A/G-antigen-antibody complexes. The tighter the binding between antibody and antigen, the more stringent the washing buffer conditions should be. The washing buffers described are used if low stringency conditions are appropriate. If higher stringency is required, increase salt concentration and ionic strength by using 0.5 M NaCl or 0.5 M LiCl for the first wash. Additionally, SDS (final concentration 0.1%) may be applied in cell lysis and the first two washes.

5. Trouble shooting

5.1 If **no signal** appears, check the following:
Sample preparation and immunoprecipitation

a. To reduce risk of antigen degradation during sample preparation, include additional specific protease inhibitors.

b. Increase the concentration of the primary antibody up to 5 µg per ml.

c. For low affinity antibodies, use washing buffers with lower stringency (150 mM NaCl, no detergent)

d. Check the affinity of the primary antibody to protein A/G-agarose according to tables 1 and 2. If the affinity of the primary antibody turns out to be low, change to an appropriate matrix.

Detection

e. Has the protein been transferred properly to the membrane during blotting?
If the transfer was not efficient, especially with high molecular weight proteins, change the transfer conditions [prolong the transfer time or increase current, change to alternative transfer buffers (7, 11)].

f. High molecular weight bands are missing: increase blotting time or change the transfer buffer.

g. Check the enzyme activity of the secondary antibody conjugate. Dot different dilutions of enzyme-conjugate onto a blotting membrane and detect directly. If no signal appears, use fresh enzyme-conjugate and test in the same way. If still no signal appears, check the detection reagent.

5.2 If **signals are weak,** check the following**:**
Sample preparation and immunoprecipitation

a. To reduce the risk of antigen degradation during sample preparation include additional specific protease inhibitors.

b. Optimize the concentrations of primary antibody.

c. Prolong the incubation time with primary antibody to several hours at 2 – 8°C.

d. Prolong the incubation time with protein A/G-agarose (overnight).

e. Shorten the washing times; use washing buffers with lower stringency (150 mM NaCl, no detergent).

Detection

f. Increase the amount of protein applied to the gel.

g. Check for efficient blotting (1e).

f. Prolong the detection time.

5.3 If **background is too high,** check the following:
Sample preparation and immunoprecipitation

a. Samples with high background may need several rounds of preabsorption to remove all the proteins binding non-specifically to protein A/G.

b. Increase the washing time of the antibody-protein A/G-agarose complex after immunoprecipitation. Increase the stringency of washing conditions as described in section 4.5.

c. Increased levels of background signals on the blot might be caused by the non-specific trapping of proteins during centrifugation of protein A/G-agarose/antigen complexes. This can be avoided by gravity-sedimentation of the complexes instead of centrifugation.

Detection

d. Use clean equipment, freshly prepared buffers and new membranes.

e. Dilute the protein concentration in the sample.

f. Avoid touching the membranes; use gloves and blunt-ended forceps with non-serrated tips.

5.4 If non-unspecific bands appear

Preabsorb the sample up to three times with protein A/G-agarose prior to the immunoprecipitation steps. If the serum immunoglobulins cannot be entirely removed during protein A/G-agarose preabsorption, serum-free cell culture conditions prior to cell lysis are recommended. Alternatively, protein A/G-agarose can be preloaded with the desired amount of specific antibody and the remaining protein A binding sites can be blocked with non-unspecific control antibodies or serum.

6. References

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* available from Roche Diagnostics

Changes to previous version Editorial changes.

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7.Ordering Information

Product	Pack size	Cat. No.
Cellular Labeling and Immunoprecipitation Kit	1 Kit	11 647 652 001
Protein A-agarose	2 ml	11 719 408 001
Protein A-agarose	5 ml	11 134 515 001
Protein G-agarose	2 ml	11 719 416 001
Protein G-agarose	5 ml	11 243 233 001
BM Chemiluminescence Blotting Kit (Biotin/Streptavidin)	1 Kit For 1000 cm^2 of membrane	11 559 460 001
BM Chemiluminescence Blotting-Substrate (POD)	1 Kit For 1000 cm^2 of membrane For 4000 cm^2 of membrane	11 500 708 001 11 500 694 001
Streptavidin-POD conjugate	500 U	11 089 153 001
Streptavidin-AP conjugate	1000 U	11 089 161 001
BM blue POD substrate, precipitating (TMB solution, ready-to-use)	100 ml	11 442 066 001
BM purple AP substrate, precipitating (BCIP/NBT solution, ready-to-use)	100 ml	11 442 074 001
Blocking reagent for nucleic acidhybridization and detection	50 g	11 096 176 001
PVDF-Western Blotting Membrane	10 sheets á 15 ×15 cm 1 roll 26.5 cm × 3.75 m	11 722 034 001 11 722 026 001
Aprotinin	10 mg 50 mg 100 mg	10 236 624 001 10 981 532 001 11 583 794 001
Complete Protease Inhibitor Cocktail Tablets	20 tablets (for 50 ml each)	11 697 498 001
Leupeptin	5 mg 25 mg 50 mg 100 mg	11 017 101 001 11 017 128 001 11 034 626 001 11 529 048 001
Pefabloc SC (AEBSF)	100 mg 500 mg 1 g	11 429 868 001 11 585 916 001 11 429 876 001
Pepstatin	2 mg 10 mg 50 mg	10 253 286 001 11 359 053 001 11 524 488 001
PMSF (Phenylmethylsulfonyl fluoride)	1 g 10 g 25 g	10 236 608 001 10 837 091 001 11 359 061 001
Micr-O-protect	10 ml	11 585 720 001
Tris	100 mg 500 g 1 kg	10 127 434 001 10 708 968 001 10 708 976 001
Sodium deoxycholate (Deoxycholic acid)	5 g	11 332 597 001
Nonidet P40 (NP40)	5 ×10 ml	11 332 473 001

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