Benchmarks


We thank Dr. J. Forstová for critical reading of the manuscript. This work was partially supported by Grant No. 20107 from Charles University, Prague, Czech Republic. Address correspondence to Zdena Palková, Department of Genetics and Microbiology, Charles University, Viničná 5, 12844 Prague 2, Czech Republic. Internet: zdenap@prfdec.natur.cuni.cz

Received 14 August 1995; accepted 7 May 1996.

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Immunopurification of Polyclonal Antibodies to Recombinant Proteins of the Same Gene Family


Polyclonal antibodies raised against bacterially expressed fusion proteins commonly cross-react with undesired proteins, such as: (i) bacterial antigens that are co-injected with the fusion protein or may be present in animal sera due to previous bacterial infections; (ii) proteins (β-galactosidase, MS2 RNA polymerase, glutathione S-transferase [GST]) in fusion with the protein of interest that are useful for enhancement of the immune response and/or purification schemes; and (iii) related proteins of the same gene family. These undesirable “contaminating” antibodies are likely to interfere with several immunological assays. In techniques such as the enzyme-linked immunosorbent assay (ELISA), immunofluorescence and immunohistological staining, which do not involve protein fractionation, it is difficult to distinguish between specific and nonspecific recognition. In Western blots and immunoprecipitation, cross-reacting antibodies may reveal undesirable bands with molecular weights close to those of the proteins of interest. High background and nonspecific results may lead to deceiving conclusions that can be avoided by the use of immunopurified antibodies.

Conventional methods to remove undesirable antibodies are based on adsorption to nitrocellulose filters, Sepharose beads or insoluble bacterial or cellular extracts (5). The most commonly used technique to clear anti-fusion-protein and anti-related protein members antibodies also involves nonspecific antigen binding to Sepharose beads (3). These methods are time-consuming and expensive, in addition to resulting in low yields and contaminated antisera. Recently, a new method was described (2) to prepare bacterial extracts for antibody purification, based on adsorption of anti-bacterial antibodies. This technique is important for the adequate screening of bacterial expression libraries. Here we describe a modification of this technique, which results in

![Figure 1. ELISA for original (orig) and purified (purif) anti-Fra-1 (panel A) and anti-c-Fos (panel B) antisera.](image)

Figure 1. ELISA for original (orig) and purified (purif) anti-Fra-1 (panel A) and anti-c-Fos (panel B) antisera. K537 E. coli bacteria transformed with pEXc-fos (coding for MS2-polymerase c-Fos protein) or pEXfra-1 (coding for MS2-polymerase Fra-1) were used to generate bacterial extracts for purification of anti-Fra-1 and anti-c-Fos antisera, produced against recombinant bacterial proteins in fusion with MS2-polymerase (panels A and B, respectively). ELISA plates were coated with a semi-purified protein solution (10 µg/µL) and incubated overnight at room temperature. The following proteins used were: MS2-polymerase-cFos (c-Fos); MS2-polymerase-Fra-1 (Fra-1); MS2-polymerase (MS2) and β-galactosidase (β-gal). Plates were washed and incubated for 30 min in blocking solution (0.5% BSA and 0.05% Tween® 20 in PBS). Original (orig) or purified (purif) rabbit antisera against Fra-1 protein (panel A) or c-Fos (panel B), diluted 1:400 in blocking solution, were added in triplicates and incubated for 2 h at room temperature. Antisera were removed, plates were washed in PBS and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:25 000 (Sigma Chemical, St. Louis, MO, USA). Antibody concentration was determined by measurement of absorbance at 495 nm upon reaction with 0.04% o-phenylenediamine (OPD) and 0.04% (vol/vol) hydrogen peroxide in 0.1 M phosphate-citrate solution (pH 5.0), and the reaction was interrupted with 1 vol of 2.5 M HCl. Chart shows relative recognition of each protein by original or purified antisera in three experiments (1, 2, 3). The reactivity of the pre-immune sera in relation to the antisera against the protein used as antigen is 0.15 for c-Fos and 0.02 for Fra-1.
antiserum with a higher specificity for the protein of interest by co-purification of antibodies that recognize fusion-portion moieties and cross-reacting antibodies to related protein family members. This method is particularly useful to generate specific antisera to different members of the same protein family, as is frequently found among oncoproteins coded for by oncogenes (fos, jun, myc, rel, etc.) and tumor suppressor genes (pRB, p16, etc.). The procedure described here is used to generate specific antisera towards two highly related oncoproteins, namely, Fra-1 and c-Fos (Fra-1 shares 40% of its amino acids with c-Fos) (1,4).

Insoluble bacterial extracts prepared by protein fixation with formaldehyde and autoclaving contain most bacterial antigens (2). When fusion proteins are insoluble and cloned homologous genes are available, the modification described here broadens the scope of this technique. To prepare bacterial extracts, instead of using bacteria of the same strain as that used for protein production, one grows bacteria transformed with plasmids coding for proteins of the same gene family as the antigen of interest, linked to the same fusion protein.

This method is being used in our laboratory to purify antibodies raised against recombinant pEX (MS2-polymerase)-fusion proteins. The procedure is as follows: bacteria transformed with plasmids coding for one family member fusion protein (e.g., MS2-c-Fos) are grown in 100 mL of LB culture medium, containing 100 µg/mL ampicillin and 60 µg/mL kanamycin, overnight at 28°C with vigorous agitation. The bacterial suspension is diluted with 300 mL of fresh LB medium, and gene expression is induced by heating bacteria to 42°C for 30 min. The suspension is further incubated for 3 h with vigorous agitation. A sample is removed for protein purification and quantification (6). The remaining suspension is then centrifuged (9000xg for 30 min), and the pellet is resuspended with 20 mL of phosphate-buffered saline (PBS) and distributed equally into two 30-mL glass centrifuge tubes. One of the tubes is autoclaved at 121°C for 1 h. Formaldehyde (0.5% wt/vol) is added to the other tube, and the suspension is incubated at 37°C for 2–24 h with vigorous shaking. Both bacterial suspensions are mixed, centrifuged (4400xg for 10 min) and washed once with 10 mL PBS. Extracts are then resuspended in 16 mL PBS, distributed into 8 plastic 50-mL centrifuge tubes (Nunc, Naperville, IL, USA) and centrifuged (4400xg for 10 min), and the pellet is stored at -20°C until use. The antiserum to be adsorbed (e.g., anti-Fra-1) is diluted 1:500 to 1:1000 in 1% bovine serum albumin (BSA) in PBS. The optimum antiserum concentration for purification using extracts produced from 400 mL bacterial suspension (about 13 mg semi-purified protein) is around 10 times that used in immunoassays such as ELISA or fluorescence-detected Western blot. Each bacterial extract pellet is resuspended with 15 mL diluted serum and incubated with mild agitation for 2 h at 4°C. After centrifugation, the supernatant is then transferred to another tube containing bacterial extract, and the adsorption cycle is serially repeated 3 more times. This immunopurified antiserum, filtered through a 0.22-µm membrane (Millipore, Bedford, MA, USA), is ready to be used. Yields vary from 26%–57%, with an average yield of 43% in 7 experiments.

For removal of anti-fusion protein only, this method can be modified by using bacteria transformed with an “empty” plasmid vector. It is also possible to decrease cross-reactivity against many different family proteins by mixing extracts containing different proteins.

Figures 1 and 2 show enrichment of antibody response against the protein originally used as antigen. Figure 1A shows the results obtained in 3 independent experiments, where anti-Fra-1 antiserum was adsorbed to a bacterial extract containing MS2-polymerase c-Fos fusion protein. The c-Fos oncoprotein displays high identity to the Fos-related antigen Fra-1 (4). Anti-Fra-1 recognition of Fra-1, c-Fos, MS2-polymerase and β-galactosidase (a nonspecific protein processed in parallel) was quantified in original and purified antiserum preparations by ELISA, and the relative absorbance values were plotted for each protein. Figure 1A shows that the degree of recognition of Fra-1 by the anti-Fra-1 antiserum increases upon treatment with c-Fos bacterial extracts.
whereas the recognition of c-Fos (a protein of the same family as Fra-1), MS2-polymerase (the protein in fusion) and β-galactosidase (a nonspecific protein that allows to control for nonspecific anti-Fra-1 reactivity as well as its reactivity to contaminating bacterial proteins) decreases. These results are confirmed by the Western blots shown in Figure 2, where anti-Fra-1 antiserum recognition of Fra-1 protein is significantly higher after immunopurification, with c-Fos recognition practically disappearing. Western blot assays also show that recognition of the MS2-polymerase protein levels decrease upon purification (data not shown). Figure 1B shows the results of purification of the anti-c-Fos antiserum by adsorption to extracts from bacteria producing MS2-polymerase-Fra-1. These results also show reproducible enrichment of the purified antibody response to c-Fos. Therefore, two specific antisera against two closely related proteins (c-Fos and Fra-1) were generated using this combined technique. These antisera are being used in our laboratory to investigate the role of c-Fos and Fos-related proteins in polyomavirus-induced malignant transformation (M.L.S. Oliveira and M.C.S. Armelin, unpublished).

REFERENCES

Quenching of Endogenous Peroxidase in Western Blot

BioTechniques 21:990-992 (December 1996)

Enhanced chemiluminescence provides a simple, sensitive, nonradioactive method for the detection of antibody binding to Western blots. Sensitive chemiluminescent substrates have been developed for use with secondary antibodies conjugated with both alkaline phosphatase and horseradish peroxidase (HRP). Similar secondary antibodies have been utilized extensively for applications in immunohistochemistry. In histochemical preparations, endogenous enzymatic activities often require pre-quenching or inhibition. In the case of alkaline phosphatase, levamisol has been used to inhibit most endogenous activities. Similarly, endogenous HRP activities are routinely quenched in immunohistochemical preparations with pre-incubation with hydrogen peroxide (H₂O₂). Similar quenching or blocking steps have not been routinely recommended for enzymatic detection in Western blots.