

Benchmarks

Immunoaffinity Purification of Antibodies against GST Fusion Proteins

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Bacterial fusion proteins have been used extensively as antigens for the generation of polyclonal antibodies. Because of their larger sizes, fusion proteins usually contain a greater number of potentially immunogenic epitopes than synthetic peptides. Standard methods for the expression and purification of fusion proteins have also added to their appeal. However, these desirable features are frequently counterbalanced by the generation of antibodies directed against the bacterial fusion partner. Although fusion partners vary in size considerably (e.g., 1–2 kDa for histidine tags vs. 27 kDa for glutathione *S*-transferase [GST]), even smaller tags can be immunogenic and seriously hamper a variety of applications, including immunodetection of target antigens, microinjection of antibodies into cells or *in vivo* administration. In such cases, immunoaffinity purification can be an effective strategy for the isolation of the desired antibody from the crude immune serum. Al-

though it is possible to immobilize the target antigen by covalent coupling onto several matrices and then bind the antibodies from the crude serum to the immobilized antigen (2), these procedures tend to be laborious and require expensive reagents. Other shortcomings include the inefficiency of the coupling reaction due to the presence of residual detergents or other impurities and ablation of potentially important epitopes. I report a simple, two-step affinity purification technique that uses GST fusion proteins immobilized non-covalently onto glutathione agarose matrices as affinity substrates.

The entire coding sequence of the Fanconi anemia complementation (FAC) group C cDNA was cloned into the *Bam*HI and *Eco*RI sites of pGEX-2TK (Pharmacia Biotech, Piscataway, NJ, USA) in-frame with the upstream epitope GST as described previously (3). The resulting plasmid, pGEX-2TK-FAC (predicted to encode a GST-FAC fusion protein of 88 kDa), and the parental plasmid were used to transform *Escherichia coli* strain HB101. A single colony of transformed bacteria grown overnight at 37°C in 10 mL of LB containing ampicillin (100

µg/mL) was used to inoculate a 1-L culture medium. After vigorous agitation for approximately 2 h (until the optical density [OD]₆₀₀ reached 0.5–0.7), the bacterial culture was induced with isopropyl β-D-thiogalactopyranoside (IPTG; final concentration 0.2 mM) and grown for an additional 4 h at 37°C. Bacteria pelleted by centrifugation (5000× *g* for 10 min) were solubilized in Buffer I (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM β-mercaptoethanol) containing 150 mM NaCl and 1.5% sarkosyl (1-g pellet per 3 mL of buffer). This protocol is a modification of one described previously (1). The bulk of recombinant GST-FAC was found in inclusion bodies, as demonstrated previously (4). Although GST alone remained soluble, for methodologic uniformity it was also isolated from IPTG-induced bacteria by the same method. Solubilized lysates were disrupted by sonication with a Branson Sonifier 250 (Branson Instruments, Westbury, NY, USA) for 1 min at power level 5 and duty cycle 50%, clarified by centrifugation at 10000× *g* for 15 min, and Triton® X-100 was added to a final concentration of 2%. A 50% slurry (wt/vol) of glutathione agarose was

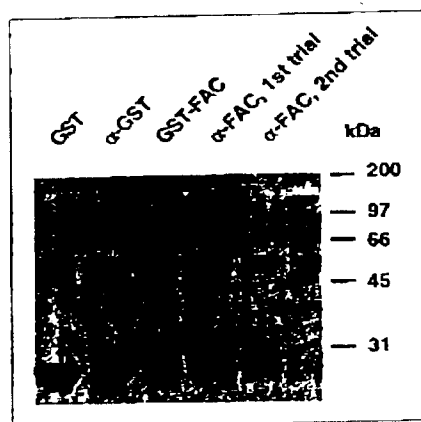


Figure 1. Detection of immunoaffinity-purified antibodies by SDS-PAGE and Coomassie blue staining. A 12% SDS polyacrylamide gel was used to analyze several steps of the purification process, including the matrices used (GST and GST-FAC bound to glutathione agarose, as indicated) and the immunoglobulins (10-µg aliquots) recovered from the GST column (α-GST) as well as from the first (α-FAC, 1st trial) and second (α-FAC, 2nd trial) uses of the GST-FAC column.

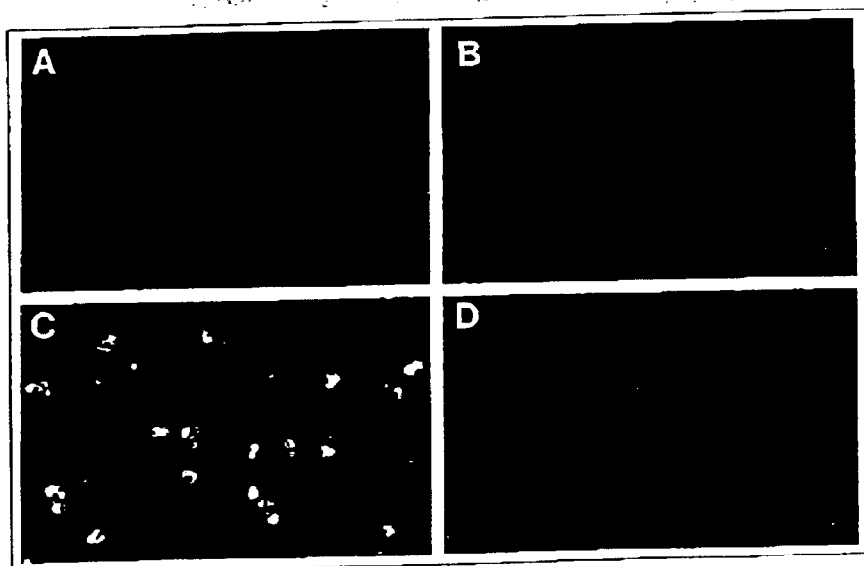


Figure 2. Subcellular location of FAC in transfected COS-1 cells by indirect immunofluorescence. COS-1 cells were transfected with pCDNA3-FAC and plated on coverslips. After 48 h, cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline. Primary antibodies used were preimmune serum (Panel A; 1:100 dilution), affinity-purified anti-GST antibody (Panel B; 1 µg/mL), affinity-purified anti-FAC antibody (Panel C; 0.1 µg/mL) and the fraction of immune serum that did not bind to the GST-FAC matrix after the first pass (Panel D; 1:100 dilution). The secondary antibody was fluorescein-conjugated goat anti-rabbit IgG (Cappel, West Chester, PA, USA). Photomicrographs were obtained at a magnification of 100× using identical exposure times (12.8 s).

Benchmarks

prepared according to the suggestions of the manufacturer (Sigma Chemical, St. Louis, MO, USA). Lysates containing GST or GST-FAC fusion proteins were incubated with hydrated glutathione agarose for 1 h by gentle rocking at 4°C. Approximately 0.5 mL of 50% glutathione agarose slurry was incubated with 12 mL of sonicate containing GST-FAC or 3 mL of sonicate containing GST. Bound agarose matrices were then washed successively with Buffer I containing 300 mM NaCl, 300 mM NaCl/15% glycerol and 150 mM NaCl.

Sera from rabbits immunized with solubilized GST-FAC (3) were used for subsequent fractionation experiments. To deplete anti-GST antibodies, 5 mL of crude immune serum were incubated with glutathione agarose coated with GST (ca. 0.5 mg) by end-on rotation for 1 h at 4°C, and the unbound fraction was collected after loading onto a polypropylene column (Bio-Rad, Hercules, CA, USA). The depleted serum was then incubated with GST-FAC (ca. 0.2 mg) immobilized on glutathione agarose for 1 h at 4°C by end-on rotation and loaded onto a polypropylene column, and the column was washed with Buffer I containing 150 mM NaCl until the OD₂₈₀ reached <0.003. For elution of antibodies, it was important to maintain the interaction of GST or GST-FAC with the agarose matrix. Preliminary experiments indicated that these interactions were stable at pH 2.8 (data not shown). Hence, the bound antibodies were eluted stepwise with 0.5-mL fractions of 100 mM glycine (pH 2.8) into 50 µL of 1 M Tris-HCl (pH 8.0) for immediate neutralization. The elution was monitored by spectrophotometry, and the extinction coefficient of IgG was used to estimate the yield of anti-GST or anti-FAC antibodies (an OD₂₈₀ of 1.33 is 1 mg/mL of IgG). Finally, glutathione agarose matrices coated with GST-FAC were regenerated by extensive washing in 100 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, and the affinity purification procedure was repeated with an additional 5-mL aliquot of serum depleted of anti-GST antibodies.

Figure 1 shows the results of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Co-

massie® blue staining of protein fractions. Acid eluates from glutathione agarose coated with GST and GST-FAC showed the expected sizes for the heavy and light chains of IgG (about 55 and 25 kDa, respectively). The total yield of anti-FAC antibodies from the first and second rounds of purification were 1.1 and 0.39 mg, respectively. In other experiments, the yield from the second round of purification was also consistently lower than the first (in the range 31%–44% of the first round).

The functional ability of anti-FAC

antibodies was demonstrated by indirect immunofluorescence of COS-1 cells transfected with wild-type FAC cloned in pcDNA3 (pcDNA3-FAC; Invitrogen, Carlsbad, CA, USA). The expected cytoplasmic localization of the protein was observed when cells were incubated with the affinity-purified antibody but not with the preimmune serum or with affinity-purified anti-GST antibodies (Figure 2). The specificity and purity of these antibodies were further demonstrated by immunoblotting and immunoprecipitation

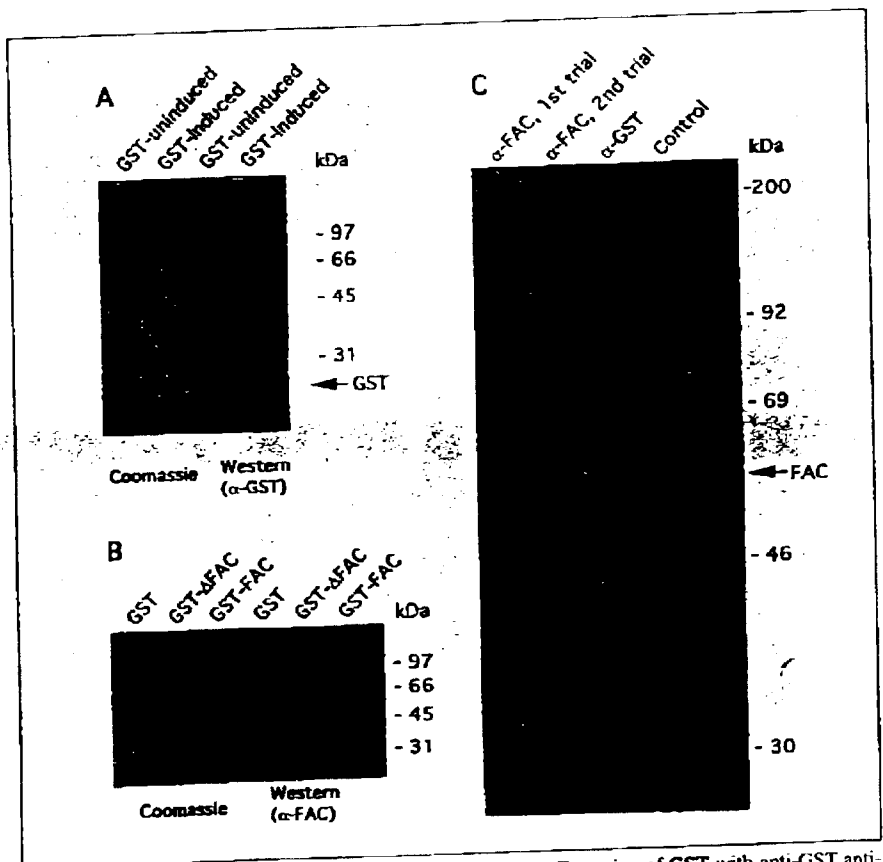


Figure 3. Characteristics of affinity-purified antibodies. (A) Detection of GST with anti-GST antibodies. Bacteria transformed with pGEX-2TK were either uninduced or induced with IPTG. Lysates from 0.5-mL cultures (OD₆₀₀ = 0.5) prepared by the sarkosyl method were incubated with glutathione agarose, washed and solubilized in Laemmli buffer (2). Eluates were resolved on a 12% SDS polyacrylamide gel followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Life Technologies) and analyzed either by Coomassie blue staining or Western blotting using anti-GST antibodies (1 µg/mL). (B) Detection and chemiluminescence (Renaissance®; NEN Life Science Products, Boston, MA, USA). Purified GST, GST fused to the amino-terminal of FAC fusion proteins with anti-FAC antibodies. Purified GST, GST fused to the amino-terminal residues, 2–303 residues of FAC (GST-ΔFAC) and GST-FAC immobilized on glutathione agarose were analyzed by Coomassie blue staining or Western blotting using affinity-purified anti-FAC antibodies (1 µg/mL). (C) COS-1 cells transfected with FAC cloned into pcDNA3 were radiolabeled with Expre-³⁵S label (0.2 mCi/mL; NEN Life Science Products) for 1 h in cysteine- and methionine-deficient medium, and cytosolic fractions (0.4 from 60-mm confluent dishes) were incubated with the indicated antibodies (1 µg per 0.4 mL lysate) as described previously (4). The control lysate was not incubated with primary antibodies. Immune complexes were precipitated with protein A agarose and analyzed by 10% SDS-PAGE and autoradiography.

Benchmarks

experiments. The expression of purified GST was significantly greater in IPTG-induced than uninduced lysates by Coomassie blue staining, as expected, as well as by Western blot analysis with anti-GST antibodies (Figure 3A). Thus, the anti-GST antibodies prepared by depletion of the immune serum are also capable of detecting the appropriate antigen. It is likely, however, that this preparation contains antibacterial antibodies. In contrast, while affinity-purified anti-FAC antibodies detected two different GST-FAC fusion proteins, these antibodies failed to detect GST (Figure 3B). Thus, the anti-FAC antibody preparation can detect the fusion partner of GST and appears to be largely free of anti-GST antibodies. Finally, these antibodies were also evaluated for their ability to immunoprecipitate FAC from transfected COS-1 cells. Incubation of cytosolic lysates successively with anti-FAC antibodies (from both rounds of purification) and protein A agarose (Affi-Gel®; Bio-Rad) followed by SDS-PAGE and autoradiography showed the expected 63-kDa antigen (Figure 3C). Similar quantities of anti-GST antibodies failed to immunoprecipitate FAC. These data demonstrate that the affinity-purified anti-FAC antibodies are monospecific and bind to soluble FAC in mammalian cells.

In summary, this protocol obviates the need for covalent coupling of the antigen to reactive matrices and can be completed in less than one day. The elution conditions preserve the integrity of the antibodies for many applications without the need for dialysis of the elution buffer. Although the subsequent binding capacity of protein-coated glutathione agarose is reduced considerably, affinity-purified antibodies obtained from the second round of purification remain comparable in purity and function to those obtained from the first round.

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Differential Amplification Kinetics for Point Mutation Analysis by PCR

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Detection of single-base mutations in DNA is a frequent task in many fields of biology and medicine. Polymerase chain reaction (PCR) has greatly facilitated obtaining fragments of DNA containing such critical nucleotide positions. However, the error-prone activity of *Taq* DNA polymerase (i.e., mismatch extension and nucleotide misincorporation), while advantageous for broadly specific amplification using partially mismatched primers, presents an obstacle when the presence or absence of an amplification product is used as an indicator for detection of point mutations. Mismatches at the 3' ends of primers, caused by critical base changes in the target DNA, do not prevent amplification of such targets because of the ability of *Taq* DNA polymerase to extend 3' mismatched primers.

Two main strategies are used to make PCR applicable to analysis of point mutations. Some methods use very low dNTP concentrations (each at 2-4 μ M), which virtually prevent mismatch extension but generate little amplification product. Thus, sensitive methods are required for PCR product visualization instead of simple agarose gel electrophoresis (4,8). Other methods artificially introduce destabilizing mismatches close to the 3' ends of primers so that an additional 3' mismatch of the primer will prevent detectable amplification even after 30-40 amplification cycles (1,3,5,9). This second approach requires extensive optimization of primers and is inherently unstable because of the possibility of additional mutations in the target DNA. It is also strongly dependent on an optimal PCR annealing temperature because the stability of primer hybridization to the target critically affects primer extension.

The kinetics of the appearance of an amplification product are influenced by any mismatch at the 3' end of a primer. During the first few cycles of a



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