

# Benchmarks

were assayed for recall proliferative response to OVA 323-339. Emulsions prepared by either the dual-syringe or sonification method stimulated a strong T-cell response, as demonstrated by the *ex vivo* proliferation assay. A likelihood ratio test indicated a stronger immunization with the sonified emulsion transferred by the method outlined above than with the emulsion prepared by the dual-syringe technique ( $P = 0.024$ ). Thus, the sonification technique produces an effective emulsion in a fraction of the time required by repeated passage between linked syringes, and the transfer technique outlined here renders this sonification easier and less wasteful.

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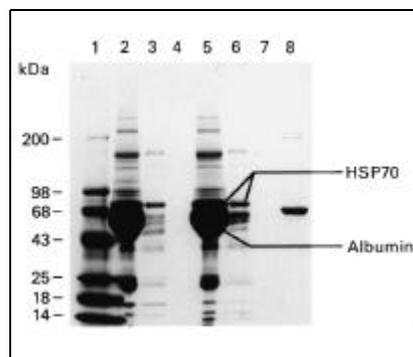
## Removal of Albumin from Multiple Human Serum Samples

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The isolation of lower abundance proteins from blood or serum is often complicated by the presence of albumin as a major contaminant. Since albumin makes up more than 60%–70% of the total plasma or serum proteins, it can mask many proteins of similar size on

gel electrophoresis. Standard methods for preparative purification of proteins, making use, for example, of differences in molecular weight, charge or isoelectric point, frequently yield limited resolution. Consequently, multiple purification steps are generally required that result in lengthy, tedious procedures and low yields, though the relevant protein may be finally adequately purified.

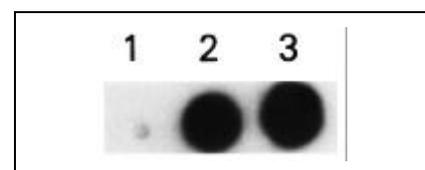
We have been interested in qualitative analysis of heat shock protein 70 (HSP 70) in the serum of Behçet's patients. Behçet's disease is characterized by severe self-limited attacks of ocular inflammation. Heat shock proteins are a ubiquitous family of proteins that play a key role in maintaining cellular homeostasis. When cells are injured or subjected to metabolic stress, synthesis of HSP is generally upregulated. We reported earlier that two proteins of 72 and 74 kDa (members of the HSP 70 family) from Epstein-Barr virus (EBV)-transformed B cells of Behçet's patients were capable of binding bovine interphotoreceptor retinoid binding protein (IRBP) peptide 1169–1191 (2,3). IRBP peptide 1169–1191 is immunopathogenic in Lewis rats (4). Hence, we wanted to analyze serum samples of Behçet's patients for the presence HSP 70. Clinical samples that were provided were limited in volume. In this report, we describe a reproducible, convenient and quick method that removes 90% of albumin from



**Figure 1. SDS-PAGE of serum proteins stained with Coomassie Brilliant Blue.** Lane 1, high mol wt standards; lane 2, original normal serum; lane 3, albumin-depleted normal serum; lane 4\*, normal filtrate; lane 5, original patient serum; lane 6, albumin-depleted patient serum; lane 7\*, patient filtrate; lane 8, commercial HSP 70 (Stressgen). (\*Filtrate is the solution that contains all proteins less than 30 kDa using the Amicon filtration unit.)

small serum samples using Affi-Gel® Blue beads (Bio-Rad, Hercules, CA, USA). Affi-Gel Blue affinity gel is a beaded, cross-linked agarose gel with covalently attached Cibacron Blue F3GA dye with a binding capacity for albumin greater than 11 mg/mL. Serum samples thus purified can be directly used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot or dot blot analyses.

The initial amount of serum used for purification was 10  $\mu$ L. Purification was carried out in 0.5- or 1-mL microcentrifuge tubes. Affi-Gel Blue beads (160  $\mu$ L) were washed with 20 mM potassium-phosphate buffer ( $K_2HPO_4/KH_2PO_4$ ), pH 7.1. Ten microliters of serum diluted with 240  $\mu$ L of phosphate buffer were added to the beads. This mixture was gently shaken on an Adams Nutator orbital mixer (Becton Dickinson, Cockeysville, MD, USA) end to end for 30-min at room temperature, then centrifuged for a few seconds to pellet the beads with the bound albumin. The supernatant was removed to a fresh tube. The pellet containing the beads with bound albumin was washed twice with 250  $\mu$ L of phosphate buffer and centrifuged briefly. The supernatants from both washes were pooled with the original supernatant. The pooled supernatant was concentrated in a Centriprep® 30 concentrator (mol wt cutoff of 30000) (Amicon, Beverly, MA, USA). This step further enriched the protein of interest because all proteins below 30000 mol wt were removed. The concentrated protein was analyzed by SDS-PAGE using a Novex electrophoresis system (Novex, Encinitas, CA, USA). The protein concentration of the samples to be analyzed were determined prior to loading on the gel. To aliquots of sample, an equal volume of Tris-glycine SDS sample buffer con-



**Figure 2. Dot-blot analysis of albumin-depleted serum.** Lane 1, commercial HSP 70 from Stressgen; lane 2, albumin-depleted normal serum; lane 3, albumin-depleted patient serum.

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taining 5% mercaptoethanol was added. Samples were incubated at 100°C for 5 min, centrifuged and then applied to an 8%–16% Tris-glycine gel (Novex). The gel was stained with Coomassie® blue. To further confirm the presence of HSP 70 in the albumin-depleted serum, dot blotting was performed. Ten microliters of samples were spotted on nitrocellulose membrane. This nitrocellulose strip was blocked for 20 min with SuperBlock™ (Pierce Chemical, Rockford, IL, USA), followed by incubation for 30 min with mAb  $\alpha$ 72/73 diluted 1:1000 (Stressgen, Victoria, BC, Canada). The membrane was further incubated with affinity-purified alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1:1000 in phosphate-buffered saline (PBS) for 30 min. After each incubation, the strip was washed with TBS (NaCl, Tris-HCl, pH 7.4) three times for 10 min each. The protein of interest was detected using the chemiluminescent detection system. After incubation with secondary antibody, the strip was washed with assay buffer (0.1 M diethanolamine and 1 mM MgCl<sub>2</sub>, pH 10) 2 $\times$ , 5 min each. The strip was incubated in 2 mL of 0.25 mM CSPD® substrate solution (Tropix, Bedford, MA, USA) for 5 min at room temperature, and then the excess substrate solution was drained. The strip was then wrapped in plastic wrap and exposed to x-ray film for 15 min and developed.

Albumin-depleted sera show significantly better resolution than undepleted sera (Figure 1). In lanes 2 and 5 of Figure 1, HSP 70 is not very clearly observed because it is masked by albumin. The HSP 70 is very clearly observed in the serum samples from which albumin was removed (Figure 1, lanes 3 and 6). Figure 2 confirms the presence of HSP 70 in the albumin-depleted samples. Previously, Burgess-Cassler described a method for removal of serum albumin in which an immunoaffinity matrix was constructed using an ammonium sulfate-precipitated fraction of goat serum (anti-human albumin) coupled to CNBr-Sepharose® (1). In comparison, our method has several advantages. First, it requires only small quantities of sample. Also, sample handling is easier in that it involves fewer manipulations with less sample

dilution. Since a very small amount of Affi-Gel Blue beads is used, there is no necessity for reusable columns or time-consuming washing and reequilibration steps. Each sample can be subjected to a fresh volume of Affi-Gel Blue.

In summary, our protocol presents several distinct advantages over existing techniques. It involves basically only two steps: 1) removal of albumin by binding to Affi-Gel beads and 2) concentration of the albumin-removed supernatant. This method can be used to remove albumin from multiple sera samples within a very short time, and the proteins of interest can be detected quickly using systems like the Phast System™ (Pharmacia Biotech, Piscataway, NJ, USA).

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