Use of High Pressure to Accelerate Antibody:Antigen Binding Kinetics Demonstrated in an HIV-1 p24:Anti-HIV-1 p24 Assay, *David J. Green, Gerald J. Litt, and James A. Laugharn, Jr.** (BioSeq, Inc., 25 Olympia Ave., Unit F, Woburn, MA 01801-6307; *corresponding author: fax 617-932-8705, e-mail jlaugharn@bioseq.com)

A major factor in the design of highly sensitive ligand-binding assays is the need to accelerate the interaction between analyte and binding partner (or capture reagent). In early immunoassay technologies, overnight incubations with exogenously supplied binding partners at low temperatures (typically 2–8 °C) were common. More recently, however, assay results are frequently required, for both medical and commercial reasons, within a relatively short time (~1–2 h). The need to accelerate the binding reaction is certainly becoming even more important as high-throughput instruments (where minutes are the more common time frame) become widely utilized. This problem is typically addressed in commercial instruments by adding a large excess of the exogenous binding partner or using less than optimum temperature conditions so as to drive the binding reaction as far as possible in an acceptable assay time.

High hydrostatic pressure is a powerful tool for studying the structure and function of proteins *[1, 2]*. Very high pressures cause most proteins to denature because of irreversible changes in the secondary and tertiary structure *[3]*. At less than the denaturation pressure, the tertiary and secondary nature of proteins are reversibly affected by changes in hydrostatic pressure *[3]*. Although some commercial applications of high hydrostatic pressure in the field of biotechnology have been reported or proposed *[4, 5]*, its use to control or modulate biomolecular interactions of commercial interest has received little attention to date. Here, we describe the use of high hydrostatic pressure to considerably accelerate the kinetics of the binding of antibody to an antigen.

Recombinant HIV-1 IIIB gag p24 (HIV-1 p24) and rabbit anti-p24 HIV-1 IIIB IgG (anti-HIV-1 p24) were purchased from ImmunoDiagnostics. A solid-phase ELISA for detecting antibody to HIV-1 p24 was developed in-house. Polystyrene microtiter plates (HiBind; Corning/Costar) were coated with a 1 mg/L suspension of HIV-1 p24 antigen overnight at 4 °C in $NaHCO_3$, pH 9.2. Unreacted sites were blocked with SuperBlock in phosphate-buffered saline (PBS; 150 mmol/L NaCl, 162 mmol/L Na_2HPO_4, 38 mmol/L NaH_2PO_4, pH 7.4)–Tris (Pierce Chemical Co.). Binding of anti-HIV-1 p24 to immobilized HIV-1 p24 was detected by using goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP; Pierce Chemical), and the HRP substrate 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS).

Experiments to determine the effect of pressure on antibody–antigen binding kinetics were commenced by adding 125 μL of 0.2 mg/L HIV-1 p24 to 125 μL of 2 mg/L rabbit anti-HIV-1 p24 in PBS in a polypropylene microcentrifuge tube such that 100 μL of the antigen/antibody mixture contained 10 ng of antigen and 100 ng of antibody. Part (120 μL) of the reagent mixture was then inserted into a deformable plastic capsule *[5]* and overlaid with melting point bath oil (Sigma). The capsule was immediately placed in the reaction chamber of a high-pressure apparatus (High Pressure Equipment Co., Erie, PA) maintained at ambient temperature (~22 °C). The pressure was then immediately increased to the desired value by means of a manually operated piston *[5]*. Control samples, in which either the antibody or the antigen was omitted, were subjected to the same experimental conditions. All experiments were performed at ambient temperature (~22 °C).

After high pressure had been applied for the desired time, test samples were measured with the p24-coated microwell ELISA. Test sample (100 μL) was immediately removed from the capsule and placed in a well of the p24-coated microplate; 100 μL of nonpressurized test solution was tested in parallel. Test samples were shaken at ambient temperature in the microtiter wells for 1 h at room temperature and then washed three times with PBS containing 0.5 mL/L Tween-20 (PBS-T). The microplates were then shaken for 1 h at ambient temperature with 100 μL of a 1:2500 dilution of the goat anti-rabbit–HRP conjugate. The microtiter plate wells were then washed five times with PBS-T, 100 μL of ABTS was added to each well, and the absorbance was read at 405 nm after a 30-min incubation.

To select appropriate reagent concentrations for experiments at high pressure, we performed an initial study with different ratios of antibody to antigen at atmospheric pressure. Antibody and antigen were mixed in polypropylene microcentrifuge vials, then held overnight at 4–6 °C to reach equilibrium binding before measurement in the ELISA. This study showed that (*a*) 100 ng of the anti-HIV-1 p24 antibody alone (with no p24 antigen) had an absorbance of ~1.1 *A* at 405 nm, and (*b*) binding of 10 ng of p24 antigen to the antibody during the overnight incubation resulted in nearly maximal inhibition of the subsequent binding of the antibody to the p24 antigen immobilized on the microtiter plate.

From the initial data described above, we determined the kinetics of binding at atmospheric pressure in mixtures containing 100 ng of antibody and 10 ng of antigen per 100 μL. The antigen/antibody mixtures were incubated for different times at ambient temperature (~22 °C) and then assayed in the ELISA. The absorbance values measured in the ELISA were used to calculate the extent of the binding of the p24 antigen with the anti-p24 antibody in the competitive assay, the results for the antibody-only sample (highest absorbance) representing zero binding and the results for overnight incubation of the antigen with the antibody (lowest absorbance) indicating 100% binding.

At atmospheric pressure, ~25% of maximal binding between antigen and antibody occurred in 1 h, 40% in 2 h, and 65% in 4 h, rounded to the nearest 5%. The binding achieved overnight (16 h) was used as the maximum binding. The results are given ±10%, reflecting the uncertainty in estimating the degree of binding from calibration

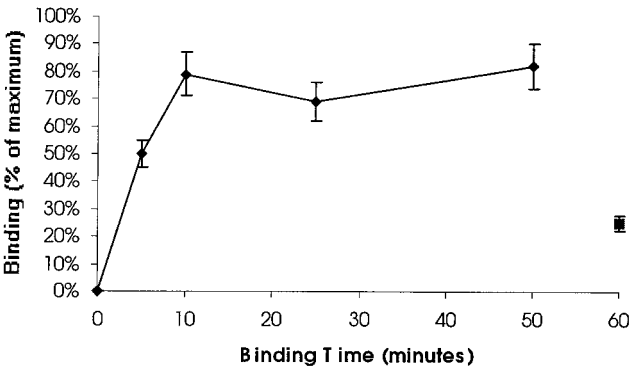

Fig. 1. Pressure-enhanced binding of p24 antigen to anti-p24 antibody. (◆) Binding resulting from pressures of 420 MPa applied for the times indicated; (■) binding achieved in 1 h at atmospheric pressure. The *bars* show the range of results for the two determinations at each time.

curves. The effects of applying different pressures for 10 min were as follows: At 70 MPa (10 000 lb./in.^{2}), no pressure effect was evident; ~10% of maximal binding was observed at 140 MPa, 20% at 210 MPa, 50% at 280 MPa, and 80% at 420 MPa. Preincubation of the anti-p24 antibody alone (without the p24 antigen) at 420 MPa for 10 min did not result in any discernible decrease in absorbance, indicating that pressures as great as 420 MPa did not affect the ability of the antibody to subsequently bind to the antigen. Applying 420 MPa for different times enhanced the binding of antigen to antibody rapidly, with 50% binding in 5 min. Maximum binding was reached in 10 min, as indicated by binding of 79% in 10 min, 69% in 25 min, and 82% in 50 min (Fig. 1). As these data show, the binding achieved in overnight incubations at atmospheric pressure was not attained at the pressures used in this study. Higher pressures than those studied here or longer incubations at lower pressures may allow greater binding to occur.

From this preliminary study we conclude that high pressure can be used to accelerate the binding of an antibody to an antigen. This phenomenon could find practical application in shortening assay times and in greatly reducing the amount of reagents required in immunoassays and other binding assays, such as those used in clinical and high-throughput screening assays.

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Acidic Citrate Stabilizes Blood Samples for Assay of Total Homocysteine, *Huub P.J. Willems,[1*] Gerard M.J. Bos,[2] Wim B.J. Gerrits,[1] Martin den Heijer,[3] Stephanie Vloet,[4] and Henk J. Blom[4]* [[1] Dept. of Hematol., Leyenburg Hosp., PO Box 40551, 2504 LN Den Haag (*address for correspondence: fax 31-70-3295046); [2] Dept. of Hematol., Dr. Daniel den Hoed Clinic, Rotterdam; [3] Dept. of Intern. Med., TweeSteden Hosp., Tilburg; and [4] Lab. of Pediatrics and Neurol., Univ. Hosp. Radboud, Nijmegen, The Netherlands]

Homocysteine is a sulfhydryl-containing amino acid, formed by demethylation of the essential amino acid methionine. Homocysteine is either transsulfurated to cysteine or is remethylated to methionine by methionine synthase. Excess intracellular homocysteine is likely to be transported to the extracellular compartment *[1]*.

Increasing evidence indicates that homocysteine is implicated in the pathogenesis of thromboembolic diseases. Several case control studies have shown a relationship between increased total plasma homocysteine (tHcy) concentrations and an increased risk of arterial *[2–4]* and venous thrombosis *[5–8]*. An increase of the tHcy concentration of 5 μmol/L is associated with 1.5–1.9 times increased risk for coronary artery or cerebrovascular disease *[9]*. These values indicate that small differences might be of clinical importance. Therefore, practical standardized conditions for handling blood specimens for tHcy determination are required. In most studies, blood is drawn in tubes containing K_3EDTA. The whole-blood sample is immediately put on crushed ice and then centrifuged as soon as possible to prevent an increase of tHcy concentrations. This tHcy increase is caused by ongoing homocysteine metabolism in blood cells, the majority of which are red blood cells *[10, 11]*. This blood handling procedure is not practical, particularly when larger studies are conducted outside a hospital setting; even in a routine clinical setting, this protocol might be hard to put into practice. To find an alternative, more suitable blood-collection medium, we investigated the effect of different blood-collection media on tHcy production when whole blood is kept at room temperature for 6 h.

Blood was drawn by venipuncture of the antecubital vein from laboratory coworkers or from consecutive patients who visited the outpatient clinics of the Leyenburg Hospital in The Hague for various reasons, unknown to the authors. Informed consent was obtained in accordance with the current revision of the Helsinki declaration of 1975. Two studies were performed. A pilot study was done with blood from 11 patients and 11 laboratory coworkers (12 men and 10 women; ages 18–63 years). Blood was drawn in tubes with 1.8 g/L K_3EDTA (Vacutainer Tube; Becton Dickinson), in tubes with 2.5 g/L sodium fluoride and 2 g/L potassium oxalate as anticoagulant (Vacutainer Tube), in tubes with 0.5 mol/L acidic citrate (Biopool Stabilyte™), and in tubes with a mixture of the sodium fluoride, potassium oxalate, and acidic citrate. Care was taken that all tubes were completely