

Benchmarks

Pressure: a novel tool for enzyme-linked immunosorbent assay procedure

Dileep Kumar Kannoujia and Pradip Nahar

Institute of Genomics and Integrative Biology (Council of Scientific and Industrial Research), Delhi, India

BioTechniques 46:468–472 (May 2009) doi 10.2144/000113118

Keywords: enzyme-linked immunosorbent assay; pressure-mediated ELISA; 1-fluoro-2-nitro-4-azidobenzene; activated surface; covalent immobilization

The enzyme-linked immunosorbent assay (ELISA) technique is the backbone of the diagnostic assay for detection of infectious and allergic diseases. Here, we demonstrate a unique ELISA method for the detection of an antigen or antibody, in which ELISA steps are carried out under pressure instead of conventional thermal incubation. Pressure-mediated ELISA (PELISA), carried out in 1 h shows more than a 2-fold increase in absorbance value than the control experiment carried out at the same time and temperature without applying pressure. Estimation of total IgE by the 1-h PELISA method gives similar absorbance value (1.081 ± 0.031 , 823.12 IU) to that obtained by 3-h heat-mediated ELISA on an activated surface (HELISA) (1.165 ± 0.037 , 810.96 IU). Since PELISA is sensitive, specific, and reproducible (intra- and interassay CVs were 6.47% and 9.65%, respectively), it could be an excellent alternative to HELISA or conventional ELISA procedures.

The enzyme-linked immunosorbent assay (ELISA) is a very useful technique for clinical diagnostics, detection of biothreat agents, high-throughput screening of drugs and drug targets, and biomedical research (1–5). ELISA is conventionally carried out by overnight incubation of an antigen or an antibody at low temperature (generally 4–8°C) on a microtiter plate through adsorption. This makes conventional ELISA a time-consuming procedure. Some commercial entrepreneurs have used large excess of the exogenous binding partner, monoclonal antibody, or sensitive tags—such as fluorescent, chemiluminescent, or radioactive moieties—to shorten ELISA timing (6–8). In other approaches, a reduction in ELISA timing was achieved by binding an antigen or antibody to an activated solid support by covalent bond rather than adsorption (9–13). Recently, Bora et al. have further shortened the ELISA timings to 3 h by doing it at higher incubation temperature on an activated polystyrene

microtiter plate (HELISA) with results comparable to conventional ELISA carried out in 18 h (14). In this communication, we report a novel and rapid method for ELISA technique in which ELISA steps are carried out under pressure.

Pressure-mediated ELISA (PELISA) was carried out on a microtiter plate that binds a protein molecule by covalent bonding. To enable covalent binding, a polystyrene microtiter plate (Greiner Bio-One, Frickenhausen, Germany) was activated by a photolinker, 1-fluoro-2-nitro-4-azidobenzene (FNAB). FNAB was prepared from 4-fluoro-3-nitroaniline (Sigma-Aldrich, St. Louis, MO, USA) by a simple diazotization reaction as described previously (15). Activation was done by coating the wells of the microtiter plate with FNAB and exposing them to 365-nm wavelength UV light as reported earlier (16). PELISA was carried out in a tightly closed 5-L pressure cooker (Hawkins Cookers Ltd., Mumbai, India). The pressure regulator from the lid was

removed and one end of a 1-m pressure tube (natural rubber, i.d. 6mm, o.d. 16 mm) was inserted through the vent pipe of the pressure cooker; the other end of the tube was passed through a pressure gauge (Laser Gasses India, New Delhi, India) and connected to a laboratory argon cylinder.

Pressure-mediated ELISA steps were optimized by doing each step at a fixed time at variable pressure and subsequent steps by conventional ELISA procedure. In optimization experiments, goat anti-human IgG (0.25 µg/100 µL 0.01 M carbonate buffer, pH 9.6/well; Sigma-Aldrich) was poured into the activated and untreated wells of a polystyrene microtiter plate and the plate was put inside the pressure cooker. A pressure of 1.0×10^5 – 3.0×10^5 Pa (measured by the pressure gauge) was applied to the closed pressure cooker by releasing argon gas and was maintained for 10 min. After the stipulated time, pressure was released slowly. Pressure-mediated blocking was optimized by adding 2% BSA (Sigma-Aldrich; 200 µL/well) and subjecting the plate to 1.0×10^5 – 3.0×10^5 Pa of pressure for 10 min. Binding of human IgG (0.25 µg/100 µL 0.01 M PBS, pH 7.4/well; Sigma-Aldrich) and anti-human IgG-peroxidase conjugate (100 µL 1:5000 v/v dilution solution in 0.01 M PBS, pH 7.4/well; Sigma-Aldrich) were optimized by applying variable pressure (1.0×10^5 – 3.0×10^5 Pa) for 10 min. After each step, the plate was washed with washing buffer (0.05% Tween-20 in 0.01 M PBS). Color was developed with the addition of 100 µL substrate-dye solution [2.5 mg o-phenylenediamine dihydrochloride (Sigma-Aldrich) and 2.5 µL H₂O₂ in 6 mL 0.2 M citrate buffer, pH 5.0]. The reaction was terminated by adding 20 µL stop solution (5% H₂SO₄) and absorbance was recorded at a wavelength of 490 nm in an ELISA reader (SpectraMax; Molecular Devices, Sunnyvale, CA, USA). All the experiments were carried out at ambient temperature (25°C ± 2). Results showed that immobilization of antigen and blocking were best achieved by applying a pressure of 1.5×10^5 Pa; however, increasing pressure up to 2.0×10^5 Pa showed a similar absorbance value (Figure 1, A and C). In contrast to other steps, applied pressure had less influence on the blocking step. This was verified by doing the blocking experiments under pressure (absorbance value 0.610 ± 0.019) and without pressure (absorbance value 0.615 ± 0.024). Similar absorbance values were found in experiments carried out with blocking and without blocking. In all these cases, appreciable change in absorbance value in negative control sera (rabbit IgG; Sigma-Aldrich) was not observed. Pressure-mediated primary and secondary antibody binding were found to be more sensitive and

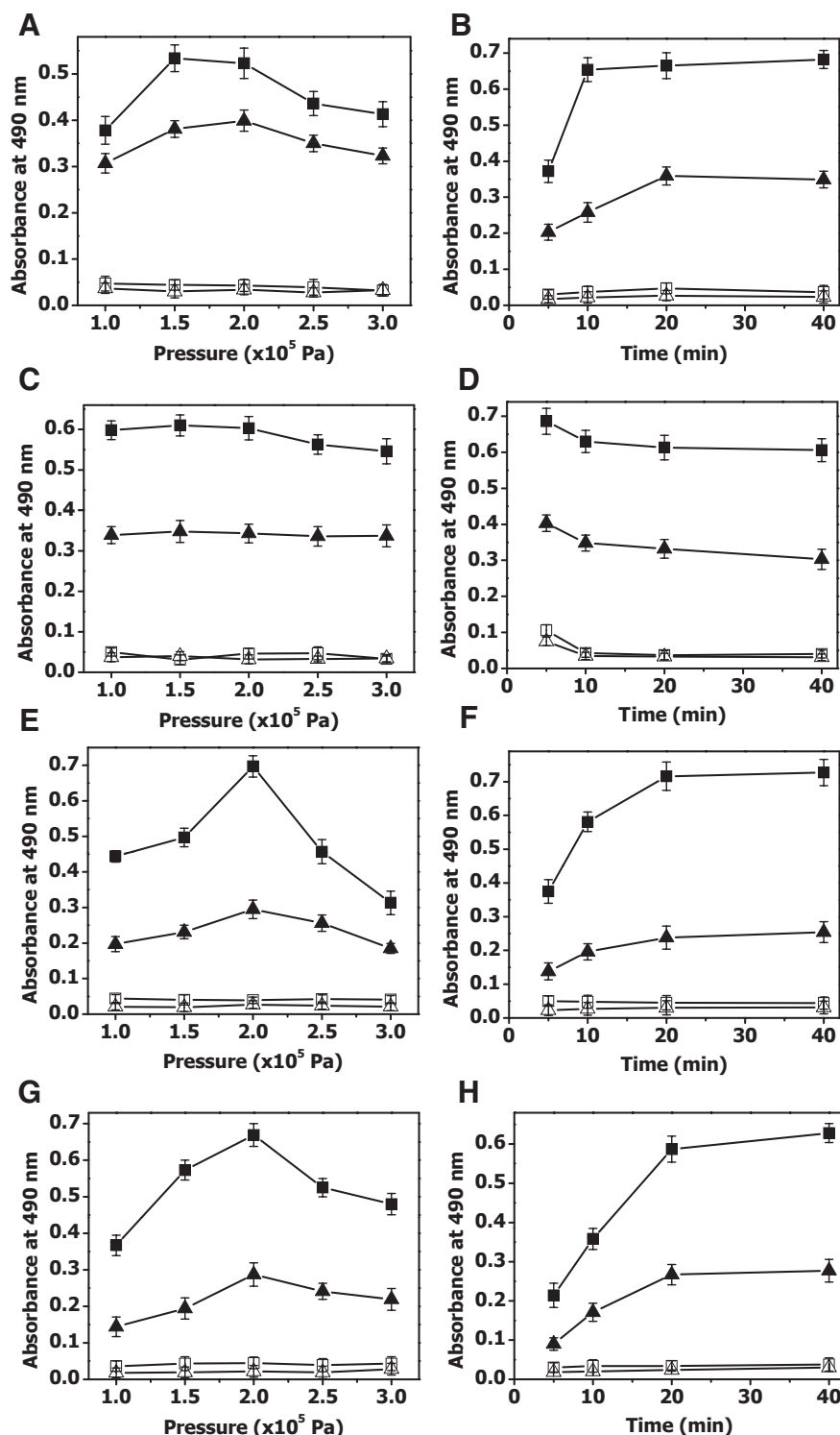


Figure 1. Effect of pressure and time on pressure-mediated ELISA steps. Immobilization of goat anti-human IgG into wells of a microtiter plate (A) at different pressures ranging from 1.0×10^5 Pa to 3.0×10^5 Pa for a fixed 10 min and (B) at a fixed pressure of 1.5×10^5 Pa while varying the time (5–40 min). The blocking step was optimized by completing it (C) at varying pressures for a fixed 10 min and (D) at a fixed pressure of 1.5×10^5 Pa while varying time periods. Optimization of human IgG binding was carried out (E) at varying pressures for a fixed 10 min and (F) at a fixed pressure of 2.0×10^5 Pa while varying time periods. Binding of goat anti-human IgG-peroxidase was optimized by doing this step (G) at varying pressures for a fixed 10 min and (H) at a fixed pressure of 2.0×10^5 Pa while varying time periods. In each of the optimization experiments, absorbance was recorded after performing the subsequent ELISA steps by conventional method. Human IgG was used as positive test sera (filled) and rabbit serum was a negative control (empty). All experiments were carried out in triplicates on the activated (square) and untreated (triangle) wells of a microtiter plate. The error bar indicates standard deviation.

both these steps require a pressure of 2.0×10^5 Pa beyond which a sharp decrease in absorbance value is observed (Figure 1, E and G). Similarly, time was optimized by doing each ELISA step at an optimized pressure at different time. Results showed that 10 min was sufficient for immobilizing antigen onto the solid surface as well as blocking, whereas maximum binding of primary and secondary antibody each occurred in 20 min (Figure 1, B, D, F, and H).

After optimizing pressure and time for each step of ELISA, we carried out all the steps of PELISA under optimized conditions and compared it with HELISA and conventional ELISA. Thus, detection of human IgG either on activated or untreated microtiter plate by 1-h PELISA (antigen immobilization for 10 min at 1.5×10^5 Pa, blocking for 10 min at 1.0×10^5 Pa, antibody binding for 20 min at 2.0×10^5 Pa, and conjugate binding for 20 min at 2.0×10^5 Pa) method was found to give absorbance value concurrent with that of 18-h conventional ELISA (11 h at 4°C, 1 h at 37°C, 3 h at 37°C, and 3 h at 37°C) or 3-h HELISA (45 min at 50°C, 40 min at 40°C, 45 min at 50°C, and 45 min at 50°C) procedure (Figure 2). However, activated microtiter plate showed ~2-fold higher absorbance value than the untreated microtiter plate. Pressure-mediated ELISA showed ~3-fold increase in absorbance value than control experiment carried out in the same time and temperature without applying pressure (Figure 2).

To generalize our method, we quantified total IgE in allergic patients' sera (obtained from Vallabhbhai Patel Chest Institute,

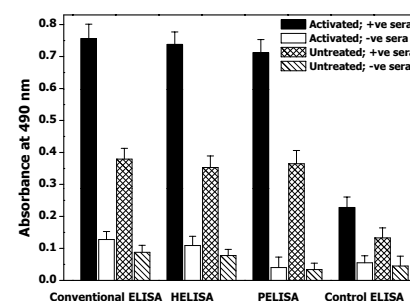


Figure 2. Comparison of PELISA with conventional ELISA and HELISA procedures. Conventional ELISA steps involve 11 h at 4°C, 1 h at 37°C, 3 h at 37°C, and 3 h at 37°C. HELISA steps involve 45 min at 50°C, 40 min at 40°C, 45 min at 50°C and 45 min at 50°C. PELISA steps are 10 min at 1.5×10^5 Pa, 10 min at 1.0×10^5 Pa, 20 min at 2.0×10^5 Pa, and 20 min at 2.0×10^5 Pa pressure. A control ELISA experiment was carried out at the same time as in PELISA without applying pressure. Human IgG was used as positive test sera whereas rabbit serum was a negative control. All the experiments were carried out in triplicates. The error bars represent standard deviation.

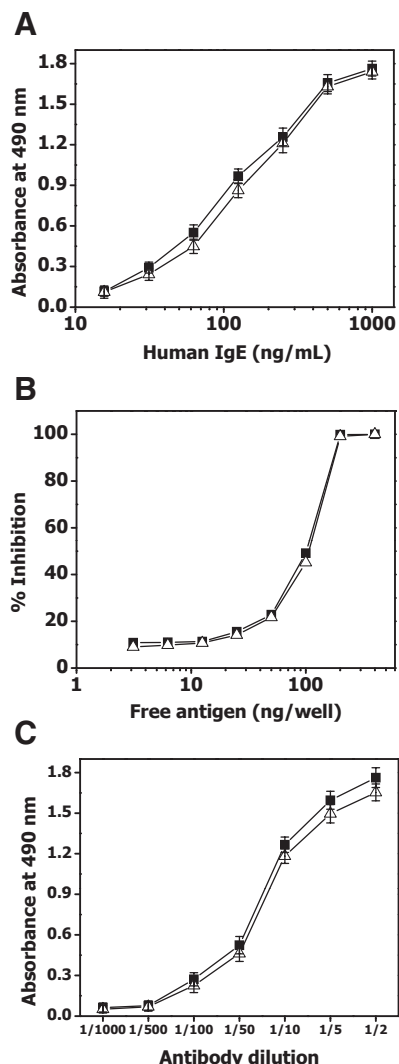


Figure 3. Studies on specificity and sensitivity of PELISA procedure. (A) Comparison between standard curves for determining IgE in human sera obtained by carrying out HELISA and PELISA using a double dilution series (1000, 500, 250, 125, 62.5, 31.25 and 15.34 ng/mL) of human IgE standard. (B) Inhibition of IgE binding to immobilized goat anti-human IgE using a double-dilution series of goat anti-human IgE (ranging 400–0 ng/well). Diluted human sera (1:10 v/v) and anti-human IgE-peroxidase conjugate (1:5000 v/v) was used in the assay. (C) Detection of IgE in diluted patients' sera (1/1000, 1/500, 1/100, 1/50, 1/10, 1/5 and 1/2 v/v dilution) by PELISA and HELISA procedure. Squares, HELISA; triangles; PELISA.

Delhi University, Delhi, with informed consent from each individual) by standard curves prepared by performing PELISA and HELISA procedure using a human IgE kit (Bethyl Laboratories, Montgomery, TX, USA). Standard curves of both the procedures were found to increase linearly with increasing concentrations of human IgE (Figure 3A). Total IgE detected in patient's sera by PELISA (1.081 ± 0.031 ,

823.12 IU) was similar to that obtained by the HELISA (1.165 ± 0.037 , 810.96 IU) procedure. Similar results obtained from both procedures suggest that the PELISA procedure did not alter the specificity of the assay. This was further confirmed by doing an inhibition study: Inhibition results both in HELISA and the present method showed similar patterns confirming that optimized pressure does not lead to any loss in assay specificity (Figure 3B). Sensitivity of PELISA was further demonstrated by detecting total IgE in pooled patients' sera. The results showed that PELISA can detect low levels of IgE similar to that of HELISA (Figure 3C).

Finally, reproducibility of PELISA method was studied by evaluating intra- and interassay CV in experiments for the detection of human IgE in pooled patients' sera. Absorbance values were expressed as the mean of three readings. Intraassay CV ($CV = SD/mean \times 100\%$) was 6.47% (48 wells). Interassay reproducibility ($CV = 9.65\%$) was determined by carrying out the same experiment fifteen times spread over 15 days.

This preliminary study highlights the potential of pressure in carrying out the important immunoassay ELISA. PELISA is found to reduce assay time to 1 h without affecting specificity or sensitivity and could be a good substitute for immunoassay procedures widely used in clinical and research laboratories.

Acknowledgments

D.K.K. thanks the University Grant Commission of India for the award of a research fellowship.

The authors declare no competing interests.

References

- Monteiro, L.L., P.S.A. Pinto, and F.S. Dias. 2006. Evaluation of the ELISA test for the antibody detection in cattle naturally and experimentally infected with *Cysticercus bovis*. *Vet. Parasitol.* 141:260-263.
- Zhao, Y.F., W.T. Jones, P. Sutherland, D.A. Palmer, R.E. Mitchell, P.H.S. Reynolds, J.P. Damicone, and C.L. Bender. 2001. Detection of the phytotoxin coronatine by ELISA and localization in infected plant tissue. *Physiol. Mol. Plant Pathol.* 58:247-258.
- Reymond, J.L. 2002. Detection strategies for catalytic antibodies. *J. Immunol. Methods* 269:125-131.
- Frankfurt, O.S. and A. Krishan. 2001. Enzyme-linked immunosorbent assay (ELISA) for the specific detection of apoptotic cells and its application to rapid drug screening. *J. Immunol. Methods* 253:133-144.

- Saita, T., H. Fujito, and M. Mori. 2004. Screening of furanocoumarin derivatives in citrus fruits by enzyme-linked immunosorbent assay. *Biol. Pharm. Bull.* 27:974-977.
- Strouse, R.J., D.W. Anderson, and D.C. Argentieri. 1991. Rapid detection of antigen-specific mouse IgE by enzyme-linked immunosorbent assay (ELISA). *J. Immunol.* 146:113-124.
- Pekary, A.E., L.F. Jr. Turner, and J.M. Hershman. 1986. New immunoassay for human thyrotropin compared with two radioimmunoassays. *Clin. Chem.* 32:511-514.
- Kemeny, D.M., D. Richards, S. Durnin, and A. Johannsson. 1989. Ultrasensitive enzyme-linked immunosorbent assay (ELISA) for detection of picogram quantities of IgE. *J. Immunol. Methods* 120:251-258.
- Bora, U., L. Chugh, and P. Nahar. 2002. Covalent immobilization of proteins onto photoactivated polystyrene microtiter plates for enzyme-linked immunosorbent assay procedures. *J. Immunol. Methods* 268:171-177.
- Zielen, S., M. Bröker, N. Strnad, L. Schwenen, P. Schön, G. Gottwald, and D. Hofmann. 1996. Simple determination of polysaccharide specific antibodies by means of chemically modified ELISA plates. *J. Immunol. Methods* 193:1-7.
- Larsson, P.H., A. Eklund, S.G. Johansson, and K. Larsson. 1997. Covalent binding of proteins to grafted plastic surfaces suitable for immunoassays: II. Picograms of IgE detected in BAL fluid in sarcoidosis. *J. Immunol. Methods* 210:41-49.
- Roch, A.M., J.G. Delcros, J.P. Ripoll, V. Thomas, J. Richard, and G. Quash. 1990. A novel covalent enzyme-linked immunoassay (CELIA) for simultaneously measuring free and immune complex bound antibodies of defined specificity. I. Application to naturally occurring antipolyamine antibodies in human sera. *J. Immunol. Methods* 133:1-11.
- Yonezawa, S., A. Kambegawa, and S. Tokudome. 1993. Covalent coupling of a steroid to microwell plates for use in a competitive enzyme-linked immunosorbent assay. *J. Immunol. Methods* 166:55-61.
- Bora, U., K. Kannan, and P. Nahar. 2004. Heat-mediated enzyme-linked immunosorbent assay procedure on a photo-activated surface. *J. Immunol. Methods* 293:43-50.
- Naqvi, A. and P. Nahar. 2004. Photochemical immobilization of proteins on microwave-synthesized photoreactive polymers. *Anal. Biochem.* 327:68-73.
- Nahar, P., N.M. Wali, and R.P. Gandhi. 2001. Light-induced activation of an inert surface for covalent immobilization of protein ligand. *Anal. Biochem.* 294:148-153.

Received 28 August 2008; accepted 16 January 2009.

Address correspondence to Dr. Pradip Nahar, Institute of Genomics and Integrative Biology (CSIR), Mall Road, Delhi, 110007, India. e-mail: pnahar@igib.res.in