Standardization of Anti-DNA ELISA Kit in Razi Institute

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Summary
In order to develop an ELISA kit for detection of anti-DNA antibodies several procedures were examined. In this study the best method for native DNA preparation, solid phase preparation, formulation of buffers, design of test performance, and a storage condition of the kit components was introduced. DNA extraction was performed by phenol procedure and purified one more time to lose protein impurities. To prepare the coated micro plate, a pre-coating stage using poly-L lysine to enhance DNA attachment and an extra post-coating stage for neutralizing its negative charge were applied. To determine the sensitivity and specificity, 120 serum samples were simultaneously tested with the developed and commercial kits. The results indicate that sensitivity and specificity of the developed kit is 97% and 100% respectively with 99% accuracy and 5% coefficient of variations. Moreover periodic examinations on kit components reveal that the kit is stable at least one year in 4°C without diminished quality.

Keywords: ELISA, anti-DNA antibody, SLE

Introduction

Detection of anti DNA antibodies in some human autoimmune diseases, such as Systemic Lupus Erythematosus (SLE) has significant importance (Stites *et al* 1997). Antibodies to native double-stranded deoxyribonucleic acid (ds-DNA) are

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particularly important because of their close association with SLE. So their presence is extremely helpful in the diagnosis of SLE and their titer correlates well with its clinical manifestations, especially renal damages (Rose *et al* 1998). Solid-phase assays, such as enzyme linked immunosorbent assay (ELISA) are the common conventional assays for anti-nDNA antibodies due to their quantitative values for a wide range of antibody activities and are easily adapted for screening large numbers of samples (Porstmann *et al* 1992). Generally, the ELISA method shows good agreement with liquid-phase methods such as the Farr assay (Johnstone *et al* 1998). The critical factors in an assay development program are reagent selection including identification of useful antibodies, label, buffers, calibrant, solid phases, and enzyme substrates. An ideal assay should always have a positive and negative reference to establish that the assay conforms and performs to a standard behavior. Finally, simplicity is the key to a successful immunoassay (Deshpande *et al* 1996).

In this study, basic principles of anti-DNA ELISA development are considered and the preparation and formulation of various reagents such as buffers, peroxidase conjugated anti-human immunoglobulin, diluents and washing solutions were studied. The optimization of DNA-coating conditions, test performance, data analysis, and handling conditions of the kit components have also been studied in our laboratory.

Materials and Methods

Extraction of calf thymus DNA. The native DNA (nDNA) extraction was done according to Cavallearo *et al* (1987) method.

Purification of nDNA. In order to remove protein impurities of both commercial and homemade calf thymuses DNA, the purification step was carried out according to Cavallearo *et al* (1987) procedure using a 25:24:1 phenol/chloroform/isoamyl alchohol mixture. nDNA integrity was already investigated acording to Bauer *et al* (1998) and confirmed by electrophoresis in comparison with Merck product

according to our previous work. Briefly, both extracted and commertial DNA preparations were digested using *ECOR*1 enzyme (Boehringer), at 37°C for 3h. Gel electrophoresis (1% agarose) was performed on cleaved samples. The purity of extracted DNA was confirmed by the comparison of bands and their similarity at molecular weights.

Preparation of the solid phase for ELISA assay. The immuno mobilization method was carried out as follows: 1) Pre-coating of the polystyrene micro titration plate with %5 poly lysine in PBS (50µl/well), with gentle shaking for 30-45min at room temperature. 2) Washing steps after discarding the poly-L lysin with 150µl/well PBS, twice. 3) Coating with 10µg/ml DNA solution prepared in TBS (10mM Tris, 150mM NaCl), incubation with gentle shaking for 2h at room temperature or overnight at 4°C. 4) Post-coating of the plate with 50µg/ml dilution of polyglotamate in PBS (50µl/well, incubation of covered plate with Para film and gentle shaking for 1h at room temperature. 5) Washing step according to the step 2 and make the plate empty, tapping it on a paper towel. 6) Saturation of uncoated sites by adding the PBS, 3% BSA, 5% fetal calf serum solution to the coated and uncoated control wells (100µl/well), incubation for 1h with gentle shaking at room temperature.

ELISA procedure. After discarding the saturation solution, 1:400 dilution of samples and control sera (from Dako standard kit) were prepared in PTB buffer (PBS containing 0.02% Tween20 and %2 BSA) and incubated in plates (100µl/well) for 1h at 37°C. The plates were washed one time with PBS plus 0.1% Tween20 and twice with PBS. After removing the washing solution, the plate was incubated for 1h with an appropriate dilution of horseradish peroxidase conjugated goat anti-human IgG antiserum (Dako), 10µl/well, washed again and allowed to incubate for 30min with an appropriate dilution of ABTS substrate prepared in MCIIvines buffer (0.11M sodium acetate, pH5.5, supplemented with 0.003%H_2O_2. After 30min, the reaction was stopped by adding an equal volume of 2MH_2SO_4 each well. Absorbance value was read at 410 nm in an ELISA reader device. In order to

determine the positive and negative results, 50 serum samples obtained from healthy subjects were examined using coated micro plates. The values were calculated and the cut off was determined. Positivity of each sample was statistically defined for OD values which were above the mean normal values±2SD (cut off). According to Deshpande (1996) samples with equal OD of cut off value were considered as questionable.

Stability tests. In order to determine the shelf life or functional stability of the kit, ten series of test reagents and coated micro plates were prepared and stored at 4°C. 25 known frozen sera that previously tested and divided in aliquots were tested by each series of kits every monthduring one year.

Assay validation. In order to determine the effectiveness of the developed ELISA for the clinical use, normal rang and predictive value of diagnostic test by defining of clinical sensitivity, specificity, and efficiency were measured. To establish the normal ranges 30 normal sera obtained from healthy blood donors were tested and the upper limit of normal sera as ±2 standard deviations was determined. In this way the value of cut off was 0.44*A* and considered as positive.

Kit examination. In comparison with the commercial kit as a golden standard (Dako Co.), about 120 unknown sera obtained from diagnostic laboratories (Noor, Danesh, and Kakh), were evaluated using the developed DNA-coated micro plate and reagents. According to the results, the true and false positive and negative values were calculated and applied to test specificity and sensitivity determination.

Results

DNA preparation. The purity of extracted DNA was compared with Merk product by the measurement of UV absorbance values at 260 and 280nm. The average A 260/280 ratios for extracted and commercial DNA were 1.72±0.01 and 1.75±0.03, respectively. To determine the consistency of results two micro plates were

separately coated with two DNA preparations. There were no significant differences between two DNA preparations ($P<0.01$), and the correlation was more than 90%.

Measurement of variation. The mean, SD, and percent coefficient of variation (CV) are commonly used as indicators of the immunoassay measurement variation. In this study one unknown serum was examined at least 10 times using different coated micro plate according to the developed procedure. To obtain the units of anti-DNA antibody/ml the OD value was measured and multiplied by 100 each time. The mean antibody unit, standard deviation, and percent coefficient of variation were 0.55, 0.03 and 5 respectively.

By consideration of commercial kit results the sensitivity, specificity and efficacy of the developed ELISA kit were calculated 100%, 98% and 99% respectively.

Stability tests. The shelf life of the kit was measured by examination of 25 known sera every month up to one year. There were no significant differences among repeated examinations in different intervals ($P<0.01$).

Discussion

Nowadays several assays have been developed to determine antibody reactivity towards ds-DNA, for instance, the anti ds-DNA ELISA is sensitive to rather low avidity anti ds-DNA and therefore detects almost all anti ds-DNA antibodies. For practical reasons as well as sensitivity reasons, the ELISA system is probably the most favored assay for the determination of antibody reactivity towards ds-DNA (Brousseau *et al* 1998).

In this study the pit falls of the anti ds-DNA ELISA system was highlighted and an antibody capture assay to measurement the level of antibody was developed. The use of an intermediate layer of poly-L-lysine to ensure adherence of ds-DNA to the plates, causes the better results. ds-DNA was used as the antigen and immobilized on a solid phase, maxisorp polystyrene microplate, then antibody from a diluted serum sample was bind to the immobilized antigen.

The uniform coating of reagents to a solid phase is key to the consistent and accurate performance of enzyme immunoassay. The CV should not exceed 5% and all binding values should be within 10% of the mean (Hansson *et al* 2001, Louzir *et al* 1992). Repeated examinations suggested that the CV is 5% in our assay. In another part of the study three lots of reagents were made and used for examination of 20 samples at the same condition. The differences between the absorbance values were less than 10% of the mean value indicated that lot-to-lot variation is not significant. It can be considered at least 0.50*A* as the positive limit to avoid false-positive results.

Moreover, we studied on different preparations of reagent formulations, such as solution, frozen, powdered, tablet, and lyophilized, to select the proper preparation of the reagents used in the assay. The results reveal that either solution or lyophilized form is the best formulation of test and both are stable at 4°C for about one year. Substrate buffer after adding the H_2O_2 and other required components should be lyophilized and kept at 4°C, but washing solution is better to be formulated as powder form sufficient for one litter. Two pooled positive and negative sera as the reference samples were considered for the developed kit assay. These samples were tested with different anti–DNA assay kits (Farr, Chitidia lucilia) and confirmed by Dako reagents, preserved in Merthiolate, which can be kept at 4°C for one year. Altogether according to stability tests and storage results we conclude that all kit components, including coated micro plate, buffers, and reference seras are stable at least one year at 4°C (unopened) and one week (opened reagents) without reduced activity.

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