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Moderate reagent mixing on an orbital shaker reduces the incubation time of enzyme-linked immunosorbent assay

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ABSTRACT

Rapid diagnostic tests can be developed using ELISA for detection of diseases in emergency conditions. Conventional ELISA takes 1–2 days, making it unsuitable for rapid diagnostics. Here, we report the effect of reagents mixing via shaking or vortexing on the assay timing of ELISA. A 48-min protocol of ELISA involving 12-min incubations with reagent mixing at 750 rpm for every step was optimized. Contrary to this, time-optimized control ELISA performed without mixing produced similar results in 8 h, leaving a time gain of 7 h using the developed protocol. Collectively, the findings suggest the development of ELISA-based rapid diagnostics.

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Emergence of the enzyme-linked immunosorbent assay (ELISA) for detection and quantification of substances such as peptides, proteins, antibodies and hormones have proven extremely beneficial in clinical laboratories because of its vast diagnostic applications [1]. A typical ELISA involves immobilization of an antigen of interest to the assay plate through direct adsorption or via a capture antibody that has been attached previously to the plate. The antigen is then detected using an enzyme labelled primary antibody (direct ELISA) or a labelled secondary antibody (indirect ELISA). A chromogenic substrate is used as an indicator of presence or absence of the antigen, wherein the color or fluorescence intensity measures the level of antigen concentrations in the assayed sample.

With the first appearance in 1971 for quantitative measurement of IgG in rabbit serum [2], the ELISA has rapidly gained importance in basic and clinical research. Though the basic principle of ELISA has remained the same, the strenuous research efforts to adapt to high-throughput and rapid operation and automation have enabled the development of miniaturized and rapid ELISA protocols. Multiplex technology using spatially separated and bead immobilization processes allows the detection and measurement of multiple targets in a single ELISA. However, the cost and availability of robotic machines for such multiplex experiments limit its application to high-end laboratories. Majority of the clinical laboratories especially those with limited resources still employ the conventional ELISA that takes 1–2 days to detect a diseased condition. Therefore such procedures are not ideal for the emergency medical conditions. The next generation diagnostics requires ELISA procedures that are simple and rapid, yet efficient and affordable to make the most use of it for rapid diagnostics.

Factors that affect the outcome of an ELISA experiment mainly include the incubation conditions, pH and ionic strength of the buffer, and concentrations of antigen and antibody [3,4]. Usually, it is the time of incubation that determines the total assay time of ELISA. Efforts to reduce the assay timing of the conventional ELISA were made using various energies and incubation strategies [5–10], assay formats and supports [11–13] and immobilization and detection strategies [14–16]. Requirement of specific instruments,

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accessories, and trained manpower are still the major limitation of many of instrumentally- advanced ELISA protocols.

In the present study, we have probed the effect of controlled shaking of antigenic reagents on the outcome of ELISA for the development of ELISA-based rapid diagnostic tests. Shaking instruments such as biological shakers and vortex mixers are commonly used for uniform mixing during biochemical reactions and incubations [17,18]. Shaking facilitates better and uniform fluid mixing, allowing more interactions among the assay components per unit time. As miniaturized shakers (e.g. microplate shaker) employ the same principle of mechanical agitation for mixing of assay components within microplate, controlled mixing via shaking may provide ideal conditions for binding interactions during an ELISA.

To probe the beneficial effect of reagent mixing on the assay timing of ELISA, we carried out the study in two stages- optimization of mixing speed and comparative assessment of the developed mixing to without-mixing ELISA protocols. Checkerboard experiments were performed to optimize the effective speed of reagent mixing during various incubations viz. coating, blocking, antigen-antibody binding and target detection. The experiments were performed in a sandwich format on 96-well polystyrene microtiter plates (Greiner Bio-One, Germany) with all incubation performed on a shaker mixer (Eppendorf ThermoMixer C, max. speed 3000 rpm, orbit- 3.0 mm) agitating at a constant speed of 0–1500 rpm. Washings between incubations were performed with PBST (10 mM PBS, pH 7.4, 0.1% tween-20) as per the conventional procedure of ELISA.

The optimization of mixing speed for individual step of ELISA was carried out by performing the rest of steps by the conventional procedure. In the first set of experiment, mixing conditions were optimized for the immobilization of capture antibody. The capture antibody (anti-human IgG, 0.1 μg/100 μL in 10 mM PBS, pH 7.4; Sigma-Aldrich, USA) was coated simultaneously onto the untreated wells as well as the 1-fluoro-2-nitro-4-azidobenzene (FNAB) treated wells of polystyrene microtiter plates [19]. The plates were then incubated for 30 min on a microtiter plate shaker working at 0, 500, 750, 1000 and 1500 rpm mixing speeds in five separate experiments. The remaining steps were performed as per the conventional procedure (blocking- 3 h, antigen binding- 3 h, detection antibody binding- 1 h; all steps at room temperature of 25 ± 2 °C). The time interval of 30 min for incubation with mixing was selected based on preliminary experiments performed for immobilization of HRP (Sigma-Aldrich, USA) onto microtiter plates. Mixing speed for BSA blocking step was optimized in the second set of experiment. For this, the capture antibody was coated on microtiter plates by overnight incubation at 4 °C (conventionally) followed by blocking incubation (2% BSA in 10 mM PBS; Sigma-Aldrich, USA) for 30 min on a microtiter plate shaker working at mixing speeds of 0–1500 rpm. The remaining steps were performed conventionally. Similarly, the antigen binding step (human IgG; 0.05 μg/100 μL; Sigma-Aldrich, USA) and target detection step (using anti-human IgG-HRP; 1:15,000 dilution; Sigma-Aldrich, USA) were also optimized for mixing speed.

As shown in Fig. 1a, the IgG detection by ELISA performed with reagent mixing on incubator shaker produced maximum absorbance read at 750 rpm. The observations were consistent for all four incubation steps of coating, blocking, antibody binding and detection antibody binding performed on both the FNAB treated and the untreated microtiter plates. This bell-shaped curve suggests the influence of reagent mixing on the outcome of ELISA. Both the low speed (≤500 rpm) and high speed (≥500 rpm) shaking of ELISA reaction mixture produced significantly low absorbance reads in 15 min as compared to those obtained at 750 rpm in same time. Shaking speed of ≥1500 rpm was found to cause spilling of reaction mixture, resulting in the error prone outcomes.

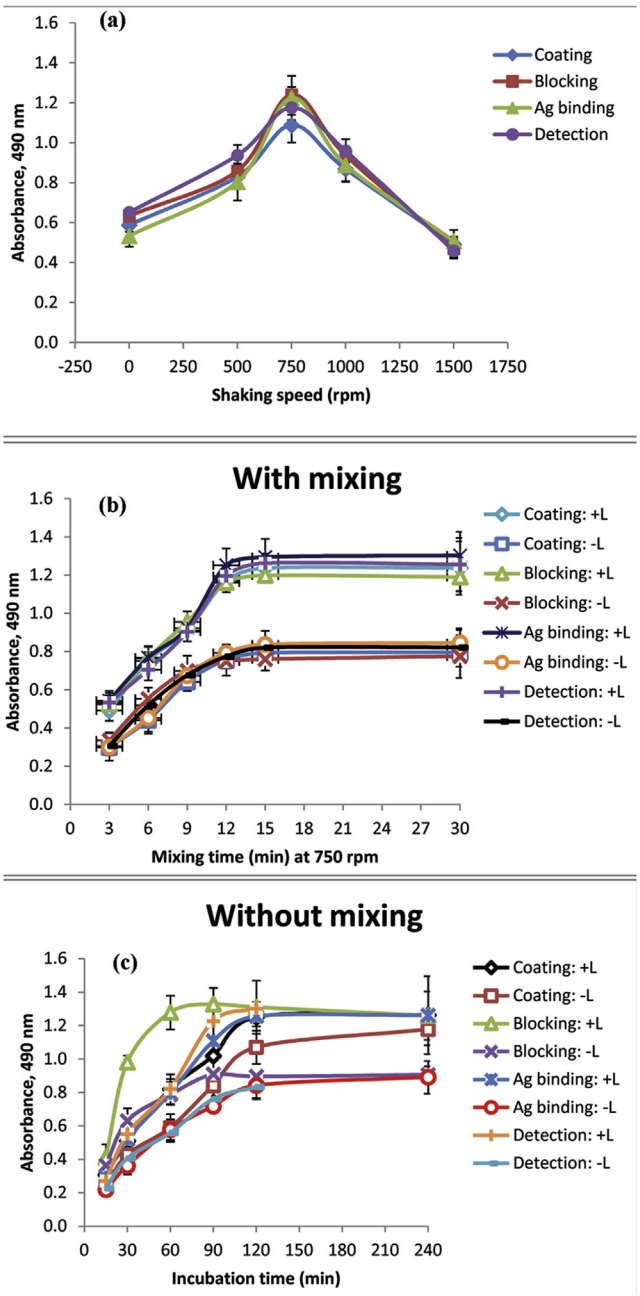

Fig. 1. Optimization of mixing speed and incubation time for rapid (mixing)-ELISA. (**a**) The effect of reagent mixing during an ELISA for detection of human IgG is studied by performing incubations at 0–1500 rpm for 30 min, while rest of steps were performed as per conventional procedure. Readings are shown for experiments performed on FNAB treated microtiter plates. (**b**) Time of incubation for rapid-ELISA is optimized at 750 rpm. (**c**) Time gain in rapid-ELISA is estimated by performing a control ELISA without mixing. The absorbance reads of test sample (human IgG) are plotted after normalizing against control sample (rabbit IgG). The + L and -L represents the FNAB-activated and untreated wells of microtiter plate, respectively. Error bars indicate standard deviation of triplicate experiment.

After optimizing the speed of mixing for various steps of an ELISA, we determined the optimal incubation time for each step of the assay. This was achieved by performing similar checkerboard experiments for antibody coating, BSA blocking, antigen binding and detection antibody binding. Incubations of 3–30 min at fixed

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