

Quartz Microtiter Plate in Homogeneous Assay Development

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Immunoassay has emerged as the most powerful tool with simplicity, specificity and sensitivity in the selective detection of various physiological, biological and environmental substances at trace levels.¹⁻³ Such methods can be classified as either heterogeneous or homogeneous. Heterogeneous arrangements are outstanding for low detection limits, but need a long reaction time compared to a homogeneous one. However, the homogeneous type assays are much faster because there is no need for the separation steps of free and bound label conjugates and, additionally, are amenable to automation.^{4,5}

Enzyme-linked competitive binding methods⁶ are based on the competition between the unlabeled analyte and an enzyme-analyte conjugate for a limited number of binding sites. The homogeneous types, such as the enzyme-multiplied immunoassay technique (EMIT), rely on the ability of analyte molecules to reverse the inhibition of enzyme-analyte conjugates induced by analyte-specific binders. The EMIT method adapted to microtiter plate assay arrangements⁷ is rarely applied, because of problems associated with non-specific adsorption of the binder and enzyme-labeled reagents to the walls of the plates. Beyond potential non-specific adsorption problem, there is also difficulty in reliably assaying certain labeling enzymes with many existing microtiter plate readers. Enzyme labels⁸ typically used in the EMIT type homogeneous assays have been two-substrate enzymes such as glucose-6-phosphate dehydrogenase (G6PDH) and malate dehydrogenase (MDH): the catalytic activity of these dehydrogenase enzymes is measured by photometric detection of NADH at 340 nm. However, the enzyme, alkaline phosphatase (AKP), can be determined with p-nitrophenyl phosphate by measuring absorbance change at 405 nm. Activity measurements at the visible region (405 nm) rather than the UV region (340 nm) could make it easier to adapt the microtiter plate reader format owing to less background absorbance from the plate materials in this wavelength region.

We adapted a quartz microtiter plate for homogeneous assay development so that it was possible to reduce the reaction volume and non-specific adsorption and to achieve a high sample throughput within a short time.

Experimental Section

Reagents and Apparatus. AKP from bovine intestinal

mucosa, N-hydroxysuccinimidobiotin (NHS-biotin), avidin from egg white, bovine serum albumin (BSA), p-nitrophenyl phosphate (p-NPP), d-biotin and all other reagents were purchased from Sigma (St. Louis, MO, USA). All chemicals used were of analytical reagent grade or better, and were used as received. All solutions were prepared using deionized water.

Enzyme activities were measured with a SPECTRAMax250 microtiter plate reader (Molecular Devices Co, Sunnyvale, CA, USA) at 405 nm. A UV transparent quartz Microtiter plate (Molecular Devices Co. Sunnyvale, CA, USA) was used.

Preparation of AKP-biotin Conjugates. AKP-biotin conjugate was prepared by reacting AKP with different amounts of NHS-biotin. The required amount of NHS-biotin dissolved in DMF was added to 500 μ L of coupling buffer containing a given amount of 200 units for AKP. The coupling buffer was 50 mM sodium carbonate, pH 9.0. The reaction was run for 24 hr at 4 °C under stirring. After reaction, conjugate was dialyzed against 50 mM Tri-HCl, pH 7.4, and diluted to a final volume of 2.0 mL with the dialysis buffer. The resulting enzyme conjugates were characterized by their residual activities and percent inhibition induced by an excess amount of avidin. The assay buffer for AKP-biotin conjugates was 50 mM sodium carbonate, pH 9.5, containing 0.01% (w/v) NaN_3 , 0.01% (w/v) gelatin, and 0.2% (w/v) BSA. Dilution of conjugates, binder, standards, and sample solutions were made using this assay buffer. All conjugates were kept at 4 °C until the additions of reagents for activity measurements.

Determination of Enzymatic Activity and Percentage Inhibition. The activity of AKP-biotin conjugates was determined by measuring the rate of appearance of p-nitrophenol, measured by the change in absorbance at 405 nm per unit time. The assay involves addition of 50 μ L of p-NPP (10 mM) and 50 μ L of appropriately diluted AKP-biotin conjugate to each well containing 150 μ L of assay buffer. For each assay, after mixing reagents, the absorbance of the mixture in each well was read over a 5 min period. In order to determine the percent inhibition value, 50 μ L of the assay buffer was replaced by 50 μ L of avidin prepared in an assay buffer. In addition, conjugates were first incubated with avidin for 5 min before subsequent addition of substrate solution.

Dose-response Curves. For the dose-response curve for biotin, 50 μ L of the biotin standard was added to each well containing 50 μ L of avidin (1.0 μ g/mL) and 50 μ L of assay

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buffer. After incubation for 5 min, 50 μL of an AKP-biotin conjugate (1 : 100 dilutions) was added, and the mixture was incubated for a further 5 min period prior to activity measurements as outlined above.

Results and Discussion

Immunoassays come in many forms. They can range from test tubes (or microtiter plate) on large analyzers, to small devices, for qualitative or quantitative assays. A trend of immunoassay tests has been in fashion of using small disposable devices that are processed on a dedicated analyzer. However, commercial disposable microtiter plates can't be used in a homogeneous assay due to non-specific binding adsorption of the reagents to the walls of the plastic plates. Here, we employed a 96-wells quartz microtiter plate instead of plastic material to prevent non-specific adsorption, and investigated the feasibility of it in homogeneous assay development. Biotin was selected as a model analyte for this study and AKP as a signal generator.

In order to perform the homogeneous assay for biotin, AKP-biotin conjugate was prepared. The particular conjugate, which prepared with a relatively high initial molar NHS-biotin: AKP ratio, therefore 2250: 1, during the conjugation reaction, possesses a residual activity of 65% and is inhibited up to 76% by an excess of avidin, was used in all subsequent homogeneous type assays.⁹ Figure 1 illustrates the effects of changing the reaction time between the AKP-biotin conjugate and avidin. As can be seen, more than 90% of the maximum inhibition, induced upon binding of avidin to AKP-biotin, was achieved within 5 min of incubation. Association rates are much faster than those observed from the heterogeneous assay system employing an immobilized binding reagent. In subsequent experiments, incubation periods of 5 min (AKP-biotin/avidin) were selected to gain a nearly maximum change in signal. Binder dilution studies were performed to determine the optimum concentration of binder for the homogeneous assay. Different amounts of avidin were incubated with a fixed amount of AKP-biotin.

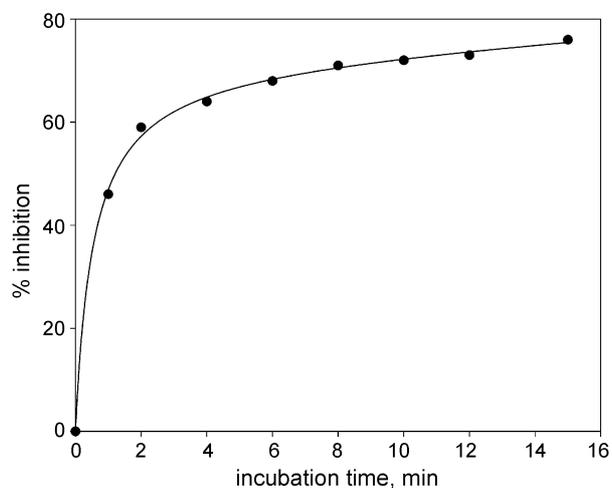


Figure 1. Effect of varying the incubation time on the percent inhibition of 0.1 units AKP-biotin induced by 2.5 μg avidin.

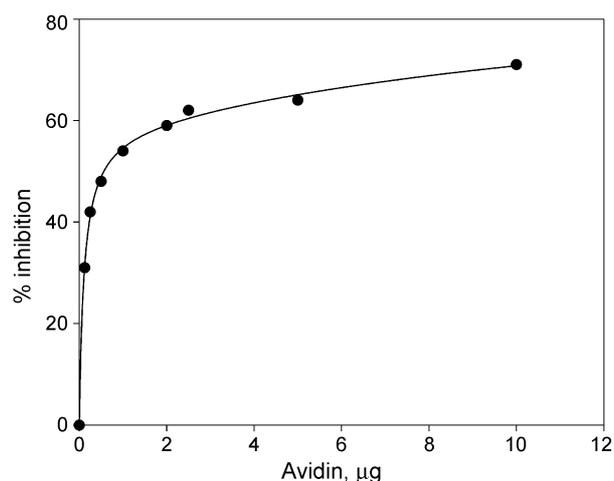


Figure 2. Effect of varying the levels of avidin on the inhibition of AKP-biotin (0.1 units).

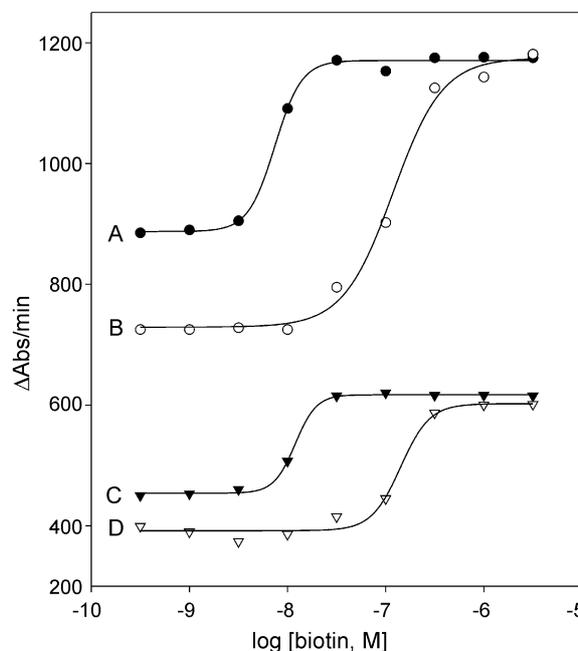


Figure 3. The effect of varying avidin and AKP-biotin concentrations on the dose-response curves obtained for biotin: (A) 0.1 units AKP-biotin and 0.1 μg avidin, (B) 0.1 units AKP-biotin and 0.2 μg avidin, (C) 0.05 units AKP-biotin and 0.05 μg avidin, and (D) 0.05 units AKP-biotin and 0.2 μg avidin.

As shown in Figure 2, the extent of inhibition increases as the amount of avidin is increased. For the AKP-biotin conjugates, the use of avidin in a range of 0.5-1.0 μg provided more than 50 % inhibition. Four different dose-response curves for biotin were constructed by varying amounts of AKP-biotin and avidin in the assay mixture (Figure 3): A) 0.1 units and 0.1 μg , B) 0.05 units and 0.2 μg , C) 0.05 units and 0.05 μg , and D) 0.05 units and 0.2 μg . As can be seen, all of the curves are relatively steep over a narrow concentration range and among them, Figure 3(A) represent the lowest detection limit. Figure 4 shows the dose-response curve for this work performed in a quartz

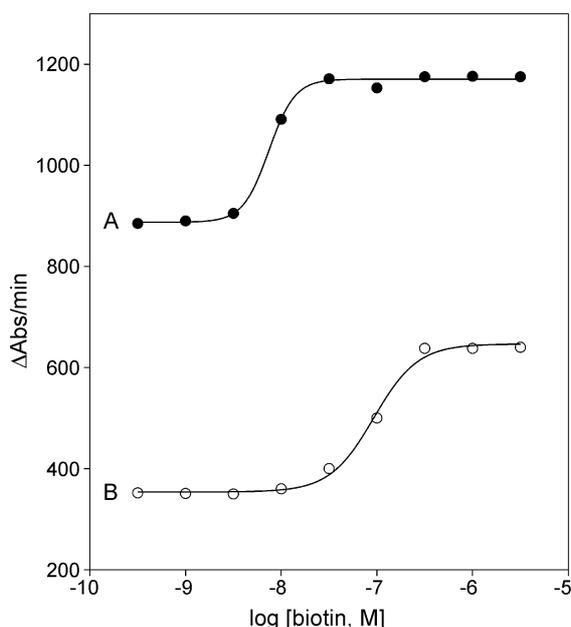


Figure 4. Dose-response curve for homogeneous biotin assay: (A) in quartz microtiter plate EMIT (0.1 units AKP-biotin and 0.1 μg avidin), and (B) in tube EMIT⁹ (0.36 units AKP-biotin and 0.40 μg avidin).

plate and compared with a previous one. Table 1 summarized the assay conditions and results for biotin. As can be seen, the detection limit in the quartz microtiter plate EMIT was found to be more than one-order lower (*i.e.*, 3×10^{-9} M of biotin, $\text{ED}_{50} = 10^{-8}$ M) when compared to the common EMIT (*i.e.*, 1×10^{-8} M of biotin, $\text{ED}_{50} = 10^{-7}$ M). Here, the quartz microtiter plate could reduce the amounts of reagents and total reaction volume. Also, it is possible to minimize non-specific adsorption. Therefore, sample throughput has been made higher, turnaround time has been lowered, and, finally, detection limit could be minimized.

Thus, the quartz microtiter plate could be used successfully in homogeneous assay development.

Table 1. Comparison of common Homogeneous Assay with Homogeneous Assay adapted to a Quartz microtiter plate, for biotin assay

	Common Homogeneous Assays ⁹	This work
AKP-biotin	0.36 units/tube	0.1 units/well
Avidin	0.40 μg /tube	0.1 μg /well
Incubation time	10 min	5 min
Total volume	1000 μL	250 μL
Throughput	1 sample/20 min	96 samples/10 min
Detection limit	10^{-8} M	3×10^{-9} M
ED_{50}	10^{-7} M	10^{-8} M

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