



The Kinetic ELISA Advantage in Quantitative Assays

Demonstrated with BioTek's[®] PowerWave[™] HT Microplate Reader and KC4[™] Software

Introduction

The ELISA technique introduced by Engvall & Perlmann (1971) and van Weemun and Schurrs (1971) attracted widespread interest as a sensitive, efficient, safe and inexpensive way to measure antigen-antibody reactions. Although there have been substantive improvements in the quality of reagents, solid phase plastics, and microplate photometers, washers, and statistical software, the basic methodology has remained virtually unchanged.

Since then several investigators have proposed that conventional ELISA may not yield truly quantitative results, leading to the emergence of a method called "kinetic ELISA" (k-ELISA).

This Application Note summarizes the favorability of k-ELISA in quantitative assays, and the successful hardware and software implementation of the k-ELISA method by BioTek[®] Instruments in its PowerWave[™] HT Microplate Reader and KC4[™] software package.

Limitations of Endpoint ELISA in Determination of Quantitative Results

The signal generating step, the reaction between enzyme and chromogenic or fluorogenic substrate, produces a chromophore or fluorophore which increases over time. Conventional ELISA adds an acid, base, or enzyme inhibitor to stop (quench) the enzyme-substrate reaction. In quantitative ELISA optical density is then determined using a photometer, and the analyte concentrations of test serum are determined from a calibration curve derived from a set of standards. This method is often referred to as endpoint ELISA.

Endpoint ELISA is based on several assumptions that have been called into question (e.g., Tsang et al., 1980):

1. Chromophore development is assumed to have ceased after addition of quenching solution. But unless well contents are mixed, chromophore concentration may continue to increase (Bullock & Walls, 1977).
2. Chromophore optical properties are assumed to remain unchanged in the interval between quenching and reading. However, the use of strong acids or bases to quench enzyme activity may shift the chromophore optical spectrum or may lead to "fading".
3. Measurements of analyte concentrations in standard solutions and test samples depend on enzyme-substrate reaction kinetics. A linear relationship between enzyme concentration and the velocity of substrate turnover only occurs when substrate is

present in great excess, and then only during the early incubation period before substrate exhaustion, pH changes, chromophore precipitation, or the build-up of inhibitory reaction products interfere with maximum-velocity substrate hydrolysis. Yet endpoint ELISA is typified by lengthy incubation periods before quenching, which suggests that the relationship between OD and concentration may not be linear over time, and that the deviation from linearity is not constant for all concentration of analyte. Endpoint ELISA often requires multiple dilutions of test serum to ensure that analyte concentration is within the linear portion of the typical sigmoid curve generated by a set of standards (Engvall and Perlmann, 1971). Single dilutions can lead to spurious estimates of concentration, especially with samples containing high analyte concentrations, due in part to a plateau of the standard curve at both high and low analyte concentrations, and in part to the so-called “hook” effect (Sorensen & Brodbeck, 1986; Vos et al., 1987).

4. Only the linear portion of the standard curve should be used to determine concentrations, which often requires arbitrary decisions as to which portion of the curve to use and reduces the dynamic range of ELISA.
5. Appropriate statistical methods for analyzing endpoint ELISA results remain controversial. Although quantitative ELISA results are usually portrayed as a relationship between optical density (OD) and $\log[\text{serum dilution}]$, statistical treatment of this relationship varies widely. This list includes: OD as a function of $\log[\text{serum dilution}]$; $\log[\text{OD}]$ as a function of $\log[\text{serum dilution}]$; OD as a quadratic or quartic polynomial function of $\log[\text{serum dilution}]$; a 4-parameter log-logistic function; weighted and simple least squares; and weighted and unweighted non-linear least squares (reviewed in Karpinski et al., 1987, who favor a 4-parameter logistic function of $\log[\text{serum dilution}]$).

Predictions and observations such as these led Tsang et al. (1980) to conclude that endpoint ELISA results should be considered only “semiquantitative”.

Development of kinetic ELISA

Tsang et al. (1980) developed k-ELISA as a method for determining concentrations of anti-*Shistosoma mansoni* antibodies in human serum. The utility of k-ELISA for large-scale diagnostic work has been explored extensively by R.H. Jacobson and colleagues (Jacobson et al., 1982; Barlough et al., 1983, 1986, 1987).

Although the early reports were based on use of polystyrene spectrophotometer cuvettes as the solid phase, the method was later modified for microplate immunoassays (Spiralnik et al., 1983; Mathews et al., 1984; Hancock and Tsang, 1986; Maddison, 1987). Use of 96-well microplates is of particular interest to laboratories with a need to analyze large numbers of samples.

Theory of k-ELISA

Substrate is added to each microplate well, and the optical density increase over time is monitored continuously. Simultaneous addition of substrate to all wells at once is desirable but not necessary, since analyte concentrations are determined by slope of the reaction curve, rather than by the absolute optical density value reached (as in end-point ELISA). If wells develop over different time periods, however, it is imperative that the slopes be determined before enzyme velocity begins to deviate from maximum (i.e. for those wells allowed to proceed for the longest intervals).

By convention, k-ELISA data are expressed as the change in milli-optical density units over time, or $dmOD/dt$. Enzyme-substrate conditions are established in order to give a change in OD during

a period of time which is convenient to the investigator, and compatible with the capabilities of microphotometers and data-capture software. Most investigators report maximum dmOD/min. values of approximately 100 for high concentrations of analyte.

The basic assumption of k-ELISA is that the enzyme molecules bound to the solid phase immune complexes catalyze substrate with maximum velocity during the entire reaction time. However, substrate exhaustion can occur near the solid-liquid interface (known as the boundary layer), especially if the enzyme concentration is high. ELISA solid phase kinetics are limited by diffusion rather than by the law of mass action (Nygren., Czerkinsky, & Stenberg, 1985; Stenberg & Stibler, 1986). Therefore, to ensure that the slope remains constant over time, stirring the microwell contents to refresh available substrate in the boundary layer is advisable.

The utility of k-ELISA also depends on the assumption that chromophore is uniformly distributed in the light path. Chromophore development occurs on the walls as well as the bottoms of microwells, but the diameter of the light path is considerably smaller than that of the microwell. Thus, optical activity may not be detected, or may seem to progress slowly, unless the contents are stirred.

k-ELISA using the BioTek Powerwave HT Microplate Reader and KC4 Software

k-ELISA conditions:

1. Nunc High-Binding microplate strips were coated with Bovine IgG reagent prepared as 1µg of BIgG/mL of NaCl solution, then dispensed 100µl/well into 96-wells.
2. Following an overnight covered and refrigerated incubation the plate was washed 3 times with the BioTek ELx405™ Microplate Washer using 300µl/well of PBS Buffer.
3. The plate was then pre-blocked with Goat Serum reagent prepared 1µg/mL of NaCl solution and dispensed 200µl/well.
4. A 1-2 hour covered and refrigerated incubation followed, and the plate was washed again.
5. The plate was then blocked with Goat Anti-Bovine IgG prepared 1µg/mL of NaCl, and dispensed 100µl/well.
6. After a covered 30-60 minutes room temperature incubation the plate was washed again.
7. Conjugation followed using Rabbit Anti-Goat IgG prepared 1µg/mL of NaCl and dispensed 100µl/well. The Rabbit Anti-Goat IgG had been pre-treated to extend storage life with reconstituted Glycerol in Deionized water in the ratio of 0.5 mg/mL of antibody to 50% glycerol solution.
8. Following a 30-60 minutes room temperature incubation the plate was washed for the final time.
9. TMB was added as the chromogen at 100µl/well.
10. The plate was then read on the PowerWave HT using KC4 software programmed for a kinetic read sequence of 30 seconds intervals over 10 minutes. Varying levels of shaking were implemented (Lo/slow (setting 1), Medium (setting 2), and Hi/Fast (setting 3)).
11. Two control plates were included in the first run, including one control plate that utilized no shaking, and a second control plate that incubated the TMB for 25 minutes before reading the plate with no shaking. This plate was run to demonstrate approximate substrate incubation time that can be saved using k-ELISA. The TMB was not quenched with a stop solution in any scenario.

Results

Fig. 1 demonstrates the significant increase in optical density (OD), which occurred if the strips were stirred with the PowerWave's shaking mode. All three of the instrument's shaking amplitudes (low, medium, and high) gave similar results. It is likely that shaking increased OD by maintaining maximum enzyme velocity and by bringing chromophore into the light path.

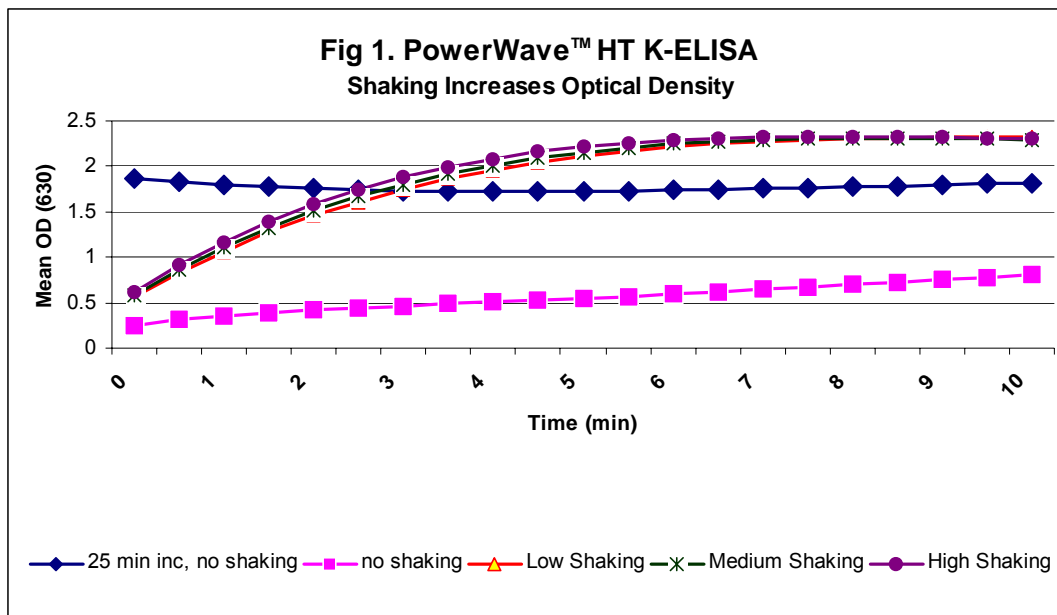


Fig. 2 shows the considerable improvement in coefficient of variation (CV%) with shaking, thus demonstrating that unequal distribution of chromophore in quiescent wells results in poor ELISA results. Again, each of the shaking amplitudes gave similar results.

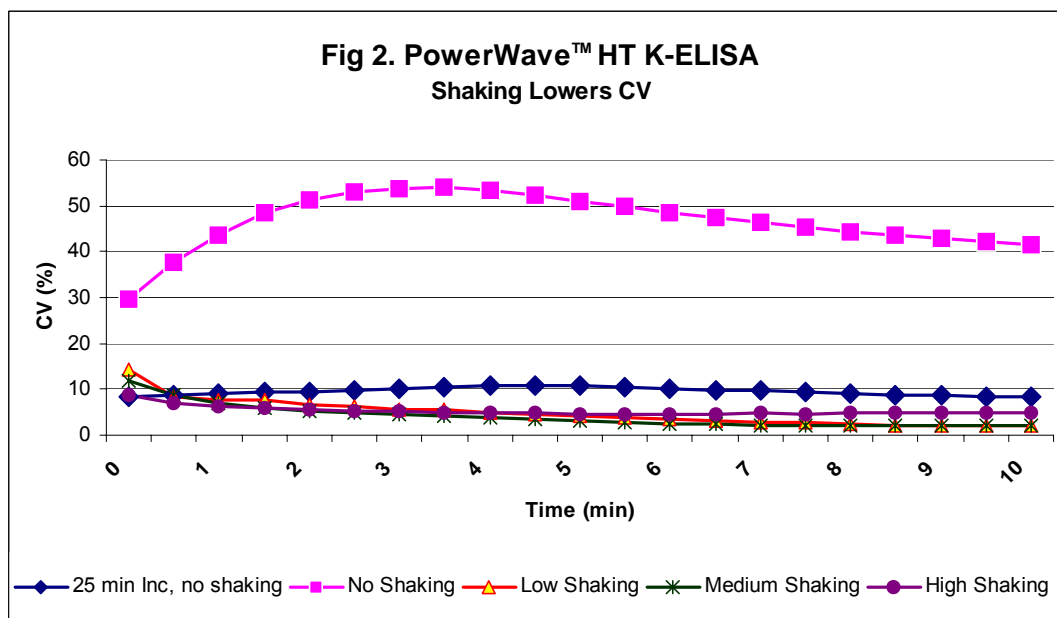


Fig. 3 shows that continuous shaking produces somewhat higher OD values than if the microstrips were shaken for only 5 seconds previous to being read. Since both continuous and intermittent agitation modes mix the well contents, it is likely that this increase is due primarily to maintaining maximum enzyme velocity.

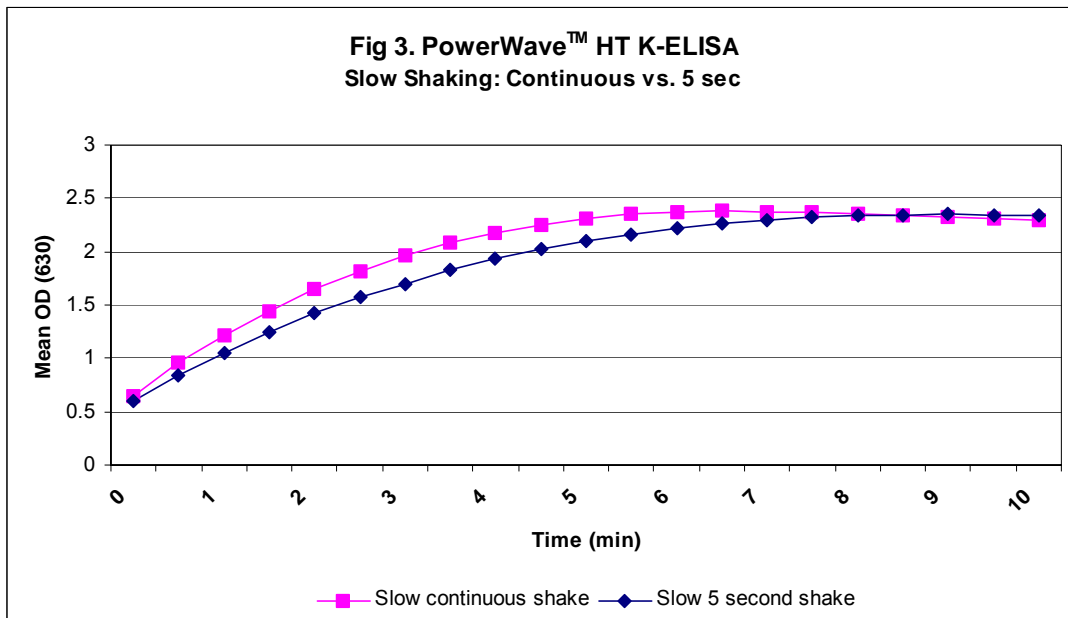


Fig. 4 shows that even with the low agitation setting, CV's are appreciably improved with continuous shaking.

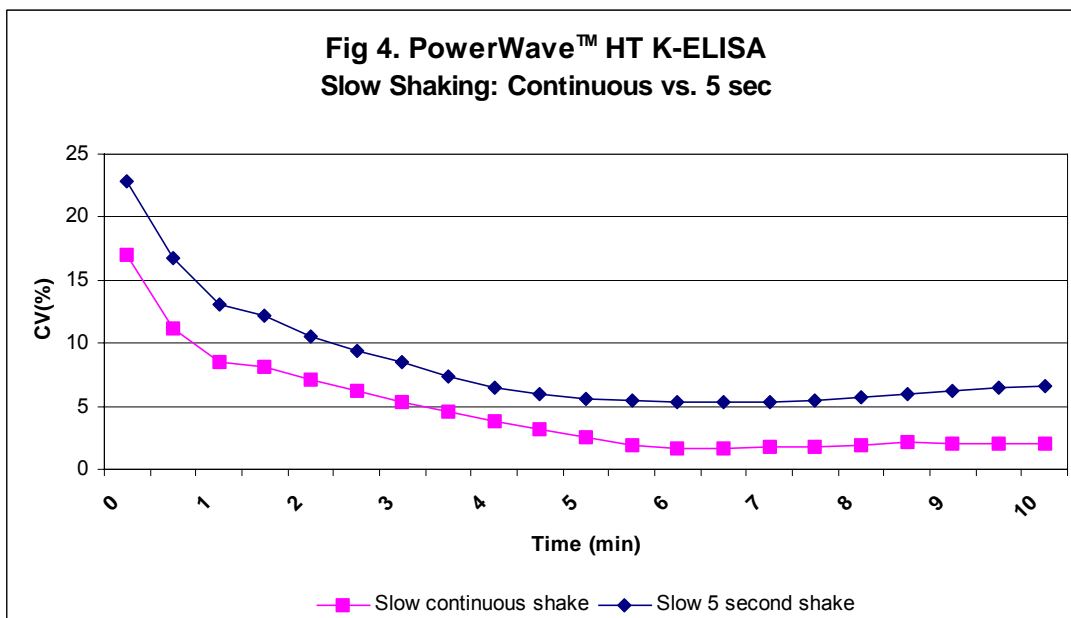


Fig. 5 demonstrates that OD values were approximately equal whether the strips were agitated continuously or discontinuously. It is likely that a 5 second agitation, using the high setting, refreshes the boundary layer and maintains maximum enzyme velocity almost as well as continuous agitation.

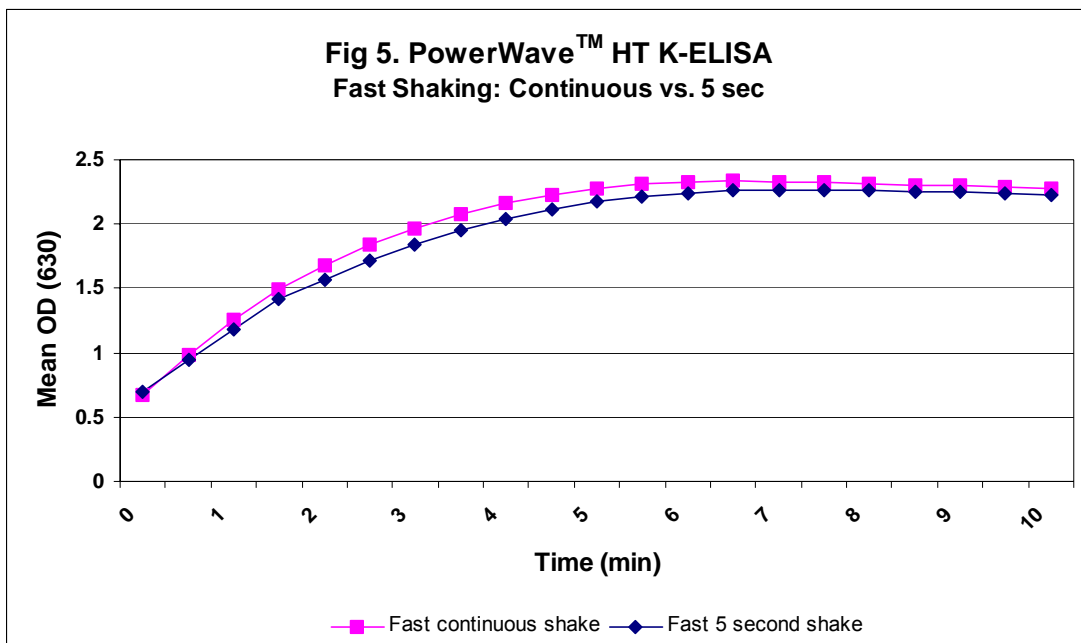
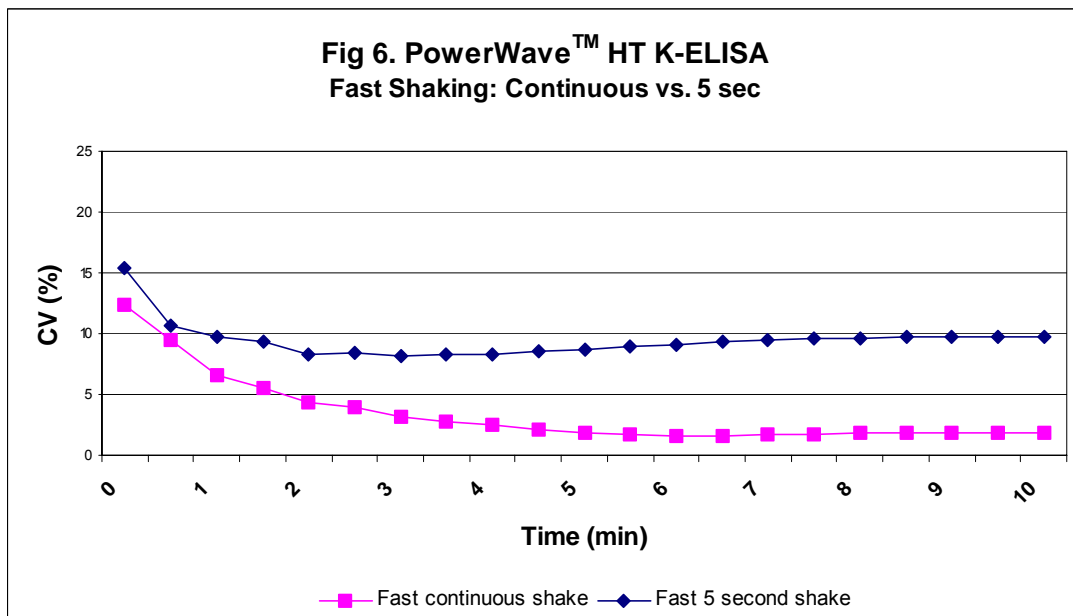


Fig. 6 demonstrates that continuous shaking, using the high setting, provides better performance in CV than intermittent shaking at the same amplitude, and slightly better performance than a continuous shake at low amplitude (Fig. 4).



Advantages of k-ELISA

- Shortens substrate incubation time by up to 25 minutes
- Eliminates quenching step
- Measures wider dynamic range of Ag or Ab concentrations
- Requires fewer multiple dilutions of unknown samples
- Allows for checking the linearity of a reaction with time
- Eliminates subjective judgment of the straight-line relationship between OD and log serum dilution
- Produces true quantitative results

Advantages of BioTek's PowerWave™ HT and KC4™ Software

- Shows lower coefficients of variation for replicates
- Produces faster results
- Shows truly quantitative results
- Eliminates quenching step by up to 25 minutes
- Requires only single dilutions
- Allows variation of shaking amplitudes to optimize results

Kinetic ELISA References

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Updated By:

Wendy Goodrich
Applications Engineer
BioTek Instruments, Inc.