Application Note

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Using the Agilent BioTek EL406 Combination Washer Dispenser to Semi-Automate a Competitive ELISA for Melamine

Semi-automated melamine quantitation

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Abstract

Melamine is a nitrogen rich compound normally used as either a flame retardant or in conjunction with formaldehyde to produce melamine resin, a durable thermosetting plastic used in the manufacture of countertops, fabrics, and glues. However, in addition to the normal use of melamine, several illicit uses for the material have been reported. This application note describes the use of the Agilent BioTek EL406 combination washer dispenser in conjunction with Agilent BioTek BioStack and Agilent BioTek liquid handling control (LHC) software to semi-automate a melamine ELISA.

Introduction

Melamine is a nitrogen rich compound that has gained notoriety as a result of its illicit use as an additive that mimics protein in foods. The practice of using melamine scrap as an additive to animal feed and food products to give the appearance of increased protein content is widespread in many countries. The basis for this are the presence of three primary amine groups, which are known to react with biuret reagent used in many of the commonly used protein assays (Figure 1). Recently, a scandal in China has implicated over two dozen companies and numerous individuals of adding melamine to milk and infant formula, leading to kidney stones and renal failure, resulting in the deaths of several infants and the sickening over 53,000 others. Trace amounts have been reported by the FDA to be in some infant formula products in the US as well.

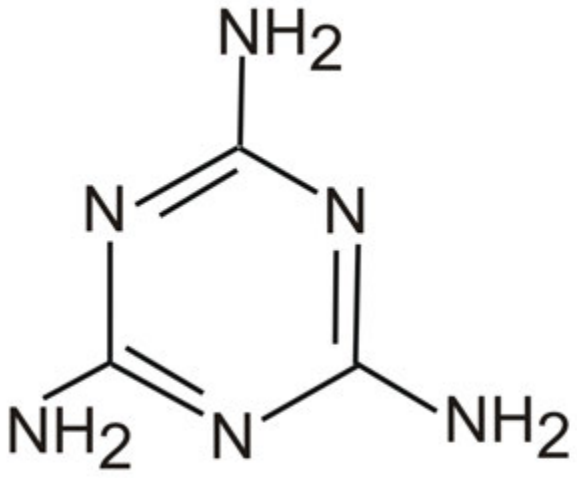

Figure 1. Chemical structure of melamine.

The melamine assay is a competitive ELISA, where melamine-HRP conjugate competes for binding to the melamine antibody attached to the wells of the microplate. Following the completion of the binding reaction, unbound sample and conjugate is removed by washing. Substrate reagent is immediately added and the color allowed to develop. The color-development reaction is terminated by the addition of stop solution and the absorbance of each well is determined. Unknown concentrations are then determined by interpolation from a standard curve generated by running standards of known melamine concentrations. To easily compare multiple experiments, the data are expressed as a ratio to the zero-standard. This ratio is often expressed as B/Bo.

Materials and methods

The melamine assay used was an ELISA kit from Abraxis (Warminster, PA) and was performed as described by the kit instructions. Samples and standards were prepared offline and 100 µL of each was pipetted manually into the assay plate. Plates were loaded into the Agilent BioTek BioStack storage stacker and the assay initiated. Plates were automatically transferred sequentially to an Agilent BioTek EL406, where 50 µL of assay conjugate was added using the peristaltic pump dispenser. The plates were then returned to the BioStack and restacked to restore their original order and allowed to complete the 30-minute incubation at room temperature. After incubation, the plates were again transferred automatically to the EL406 and washed four times with 300 µL of washer buffer followed by the addition of 100 µL of substrate solution using one of the syringe pump dispensers. Plates were automatically returned to the BioStack and restacked. The color development continued for 20 minutes. After color development, the plates were automatically returned to the EL406. A 100 µL amount of stop solution was added using the second syringe-pump dispenser and the plates returned to the BioStack. After restacking, the absorbance of each well at 450 nm was determined using an Agilent BioTek Synergy 4 multimode microplate reader.

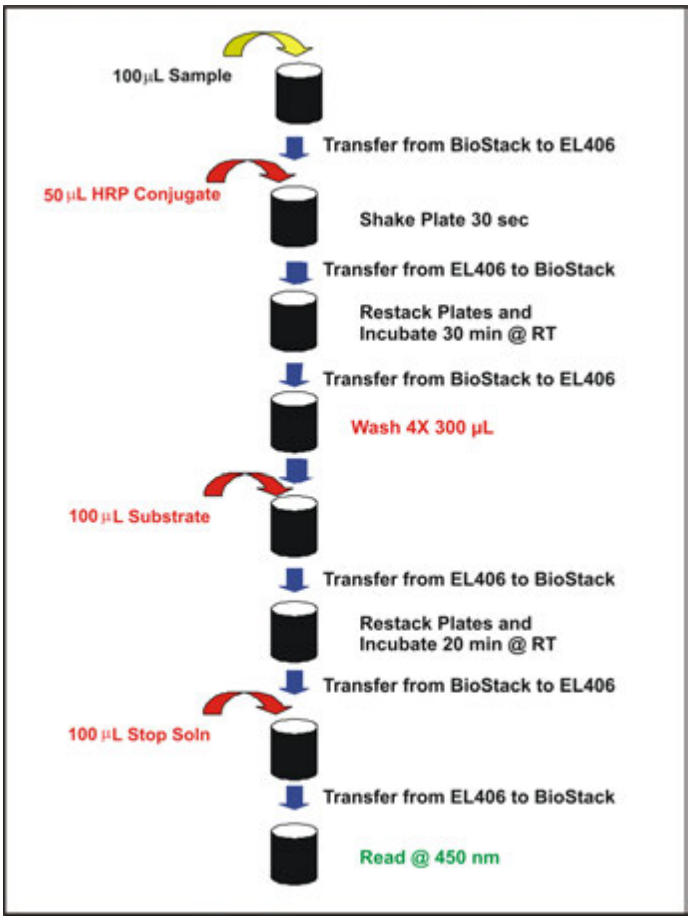

Figure 2. Schematic diagram of the procedural steps of the melamine ELISA reaction. Processes carried out by the Agilent BioTek EL406 washer dispenser are indicated in red.

This procedure was automated using LHC PC software to control the plate movement, processing, and incubation steps (Figure 3). Incubation steps were carried out in the BioStack, while plate processing, such as wash steps, shaking, and reagent addition was accomplished using the EL406. A single LHC program file was written to carry out all of the process steps after the addition of sample to the plate up to the point of reading the plate. Plates were moved to the EL406 for liquid handling steps such as wash steps to remove unbound materials or the addition of reagents (e.g. conjugate, substrate, and stop solutions). The incubation steps were carried out using the BioStack as a means to store the plates. Delay times were calculated such that the process times and restack procedures were accounted for (Figure 3).

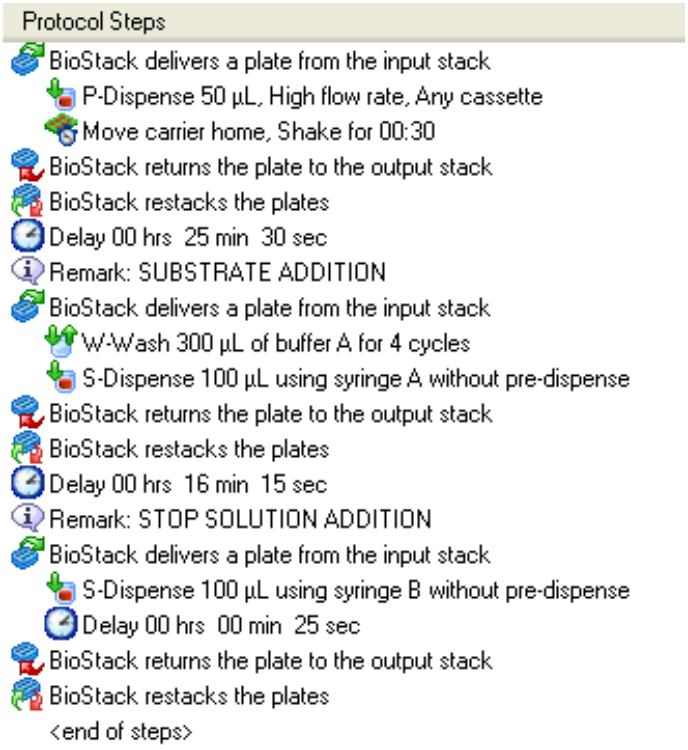

Figure 3. Agilent BioTek LHC process steps for a 3-plate assay batch.

Results and discussion

The data in Figure 4 demonstrate the efficacy of using the EL406 to automate the fluid handling tasks of the melamine ELISA. Unbound material was removed using the washer function, while the necessary reagents were added using the three dispensers of the instrument. Using a B/Bo calculation where the ratio of each standard to that of the zero-standard, significant changes in signal are observed as a function of melamine concentration.

Using a 5-parameter logistic fit of the data, unknown sample concentrations can be determined by interpolation from the curve. Detection limit calculated by interpolation from the standard curve of the mean of the zero standards minus 3 times its standard deviation, ranged from 10 to 30 ng/mL in three different experiments.

The data presented here demonstrate the ability of the Agilent BioTek EL406 to automate the liquid handling tasks required to run the melamine ELISA kit from Abraxis. The resulting data was equivalent to that provided by the kit insert as example data. EL406 can serve to provide the same functionality as four different instruments (1 washer and 3 dispensers). In conjunction with the Agilent BioTek BioStack, the EL406 can be used to automate the liquid handling processes of this assay.

The melamine ELISA has the capability to detect melamine contamination in the parts per billion (PPB) ranges. This is sufficient for many applications, as the FDA guidelines for consumption list levels above 1 ppm as being hazardous. The FDA methods employ LC/MS or GC/MS and have the advantage of being more sensitive and being able to detect cyanuric acid concurrently. However, these methods also require much more expensive instrumentation, more extensive sample preparation prior to the assay, and a higher degree of technical training of the technician carrying out the assay. In many instances, the ELISA method can be used as a screening tool when there are large numbers of samples and time to result is critical.

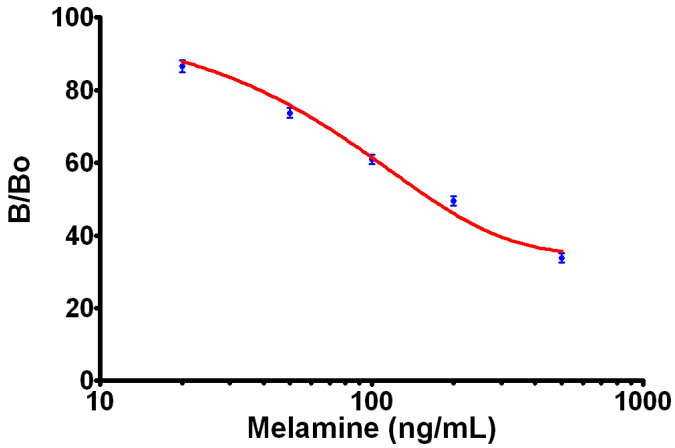

Figure 4. Melamine concentration curve. B/Bo calculations based on the zero-standard were determined using Agilent BioTek Gen5 data analysis software from three different plates. The data were plotted using GraphPad5. Each data point represents the mean and SEM of a total of 72 determinations.

Conclusion

Semi-automation of ELISA procedures such as the melamine assay allow unattended processing of a number of assay plates. While the assay is not completely “walk-away”, the time consuming liquid handling steps, such as reagent addition, washing, and incubation have been automated. After processing, the plates are read manually using an absorbance reader. Once the absorbance has been measured, B/Bo calculations, the generation of a standard curve and the interpolation of the curve for the determination of sample concentrations is automatically performed by the Agilent BioTek Gen5 data analysis software.

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